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THE UNIVERSITY OF ALBERTA

VACCINIA VIRUS STRUCTURE AND COMPOSITION

by

Ⓒ JACQUES BOISVERT

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT... MICROBIOLOGY ....

EDMONTON, ALBERTA
FALL, 1976

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled VACCINIA VIRUS STRUCTURE AND COMPOSITION submitted by JACQUES BOISVERT in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Microbiology

T. Y.........................
Supervisor

M. S. Shahrabadi

Russell L. Kelly
External Examiner

Date July 9th 1975

ABSTRACT

Whole vaccinia virus was found to be composed of 33 polypeptides of molecular weights ranging between 11,000 and 150,000 daltons, when analysed by the technique of polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

Specific staining techniques of SDS gel have indicated the possible absence of lipopeptides and the presence of 2 major glycopeptides plus 6 minor ones.

Electron microscopy examination of viral particles extracted by detergents has revealed an "S" shaped structure for the nucleoprotein complex of the virions. Evidence obtained by electron microscopy and gel electrophoresis indicated that vaccinia virus purified by sedimentation velocity in a sucrose gradient was heterogenous with respect to solubilization by detergents. Furthermore, evidence obtained also suggested that some of the polypeptides composing the virus were located in the inner and in the outer regions of the viral particles.

A major surface antigen called complex E was purified from an extract obtained with a detergent. This antigen, which is a glycoprotein, has a molecular weight estimated between 100,000 and 200,000 daltons. Neutralization experiments have shown that antibodies produced against complex E can neutralize vaccinia virus.

The immunoferritin technique has indicated a wide distribution of complex E over the outer region of the viral particles. Combined with negative contrast staining and ultramicrotomy observations, the above results have indicated that complex E is located on the surface of the viral particles.

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INTRODUCTION

STRUCTURE OF VACCINIA VIRUS

Because of its large size, vaccinia virus was one of the first viruses to be studied using the electron microscope (Green et al., 1942). Various techniques with increasing resolution were used to examine the virus.

Shadowed preparations of crude and purified viruses showed a brickshaped particle with a rough surface which was thickened or elevated at the center (Sharp et al., 1946; Dawson and MacFarlane, 1948). Its dimensions were roughly 100 x 200 x 300 nm.

The technique of negative contrast staining using various heavy salts was succesfully utilized by many authors (Nagington and Horne, 1962; Noyes, 1962a; Noyes, 1962b; Westwood et al., 1964), who demonstrated the presence of tubules or rodlets covering the outside surface of the virions in a seemingly random fashion. These studies also showed the presence of two types of particles: the M form and the C form (Nagington and Horne, 1962). The M form refers to the mature viral particles, while the C form refers to a particle somewhat larger than the M form and electron dense. Westwood et al. (1964), using negative staining in conjunction with various chemical and enzymatic treatments, found that both particles (M and C) were mature and interchangeable. The only explanation to date is that the C form represents a damaged particle which swells upon penetration by the heavy salts giving rise to the larger and electron dense C form (Westwood et al., 1964).

Ultramicrotomy has been the method of choice to elucidate the structure of vaccinia virus (Peters, 1956; Dales and Siminovitch, 1961; Dales, 1963; Peters and Buttner, 1965; Dales and Mosbach, 1968). Basically, the structure is made of three components: a central biconcave DNA containing core, flanked by two electron dense lateral bodies responsible for the central thickening seen in shadowed preparations. Finally, a trilaminar envelope surrounds those two structural components. This envelope is covered with projections or spicules which are especially well seen when the envelope covers the immature form (Dales and Mosbach, 1968; Trippier et al., 1972).

Other techniques have been used to probe the structure of vaccinia virus. Controlled degradation of the virus by a non-ionic detergent NP-40, followed by enzymatic digestion has shown that the external envelope and the lateral bodies were removed to release the DNA containing core (Easterbrook, 1966).

The fragile internal details of the core arrangement were obtained using various fixation methods such as formaldehyde, glutaraldehyde and potassium permanganate (Peters and Mueller, 1963; Peters and Buttner, 1965; Peters, 1966). Transverse sections of the core usually showed three tubes of 40-50 nm in diameter. Seen from a horizontally cut section (long axis), they appear as three tubules parallel to the long axis and joined together to form an "S" shape. These tubules are thought to be embedded in a matrix of DNA as shown by the silver Feulgen technique (Peters, 1966).

Between the core region and the lateral bodies, there is an area or membrane like structure of low electron density. Its partial sensitivity to proteolytic enzymes demonstrated it protein nature (Peters, 1956; Peters, 1960).

More recently, other techniques have been used to show different aspects of viral structure. Although freeze-etching does not have the resolution of ultramicrotomy or negative staining, it permits topographical views of the virus (Medzon and Bauer, 1970; Easterbrook and Rozee, 1971; Easterbrook, 1972). The immature forms of the virus, as seen in the cytoplasm of infected cells, were shown to posess sub-units on their surface. These can be correlated to the spicules seen in thin sections.
The information obtained by examining cleavage planes of mature viral particles has indicated that the core surface has a pebbled appearance and the removal of the lateral bodies leaves a depression in the middle of the core (Medzon and Bauer, 1970).

Freeze-etching studies have also revealed that the sub-unit arrangement covering the trilaminar envelope of the immature forms appear to reorganize into a more tightly packed structure of the mature forms (Medzon and Bauer, 1970; Easterbrook, 1972).

COMPOSITION OF VACCINIA VIRUS

Chemical analysis of vaccinia virus has revealed the following composition: 5% DNA, 1.2% cholesterol, 1.7% fatty acid, 2.1% phospholipids (mainly lecithin), 0.2% non-DNA carbohydrates and about 90% protein (Hoagland et al., 1940; Smadel et al., 1940; Smadel and Hoagland, 1942; Joklik, 1962a; Zwartow, 1964; Joklik, 1966; Dales and Mosbach, 1968; Woodson 1968).

The presence of other materials has been associated with vaccinia virus, but was considered to be contaminant. The most interesting one is RNA, which was found in highly purified preparations (Joklik, 1962a; Planterose et al., 1962). Even after treatment with RNases, the virus still contained 0.2% RNA. It was judged as a contaminant non specifically packed inside the virus during the process of maturation.

Glucosamine constitutes more than 90% of the carbohydrate present in the virus, while galactosamine represents 10% (Holowczak, 1970; Garon and Moss, 1971). Most of the glucosamine (93%) was associated with the viral external envelope (Holowczak, 1970).

The external envelope enclosing the virus was shown by Dales and Mosbach, (1968) to have all the characteristics of a unit membrane. Their analysis showed that 85-90% of the total phospholipids was present in this envelope. Their studies using labelled lipid precursor also showed that since not all the radioactive lipids could be removed when the external envelope was extracted and that cores did contain some labelled material, it could very well be located in the light area or membrane like structure that encloses the DNA matrix. This light area has been commonly called the core envelope.

Many techniques for the extraction of viral DNA have been used. Sodium dodecyl sulfate extraction followed by pronase digestion is the preferred method. The calculated molecular weight of the genome was found to be $160\text{x}10^6$ daltons (Sarov and Becker, 1967). Electron microscopic examination of the DNA has revealed a linear duplex having a contour length of 87 nm (Easterbrook, 1967) which corresponds to the molecular weight generally accepted (Joklik, 1962c).

The protein constituents of vaccinia virus can be studied in two different ways: first, as aggregates or structural components like the lateral bodies or the core etc.; second, they can be studied as individual proteins or polypeptides composing these structural components.

The complete release of the protein constituents of vaccinia virus has been difficult because of the relative resistance of the virions to enzymatic, chemical and mecanical disintegration. The number of antigenic components was found to vary between 8 and 20, when soluble antigens or disintegrated viruses were analysed by Ouchterlony gel diffusion or immunoelectrophoresis (Marquardt et al., 1965;

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Westwood et al., 1965; Zwartow et al., 1965; Rodriguez et al., 1966).

No accurate number of structural proteins can be given since the analysis of the soluble antigens will reveal components which although virus-coded are not structural components. Analysis of the disintegrated viruses will not reveal all the antigens because they are not released from the virus or they are destroyed by the treatments used to break up the viral particles.

The study of vaccinia virus soluble antigens has indicated that they could be divided into two size groups. The first group contains antigens with a molecular weight between 50,000 and 100,000 daltons while the second contains antigens with a molecular weight in excess of 200,000 daltons. Both groups contain viral structural antigens. The second group also contains the antigen(s) responsible for eliciting neutralizing antibodies (Cohen and Wilcox, 1966). Appleyard et al. (1964) also using gel chromatography separation of soluble antigens, found a molecular weight of slightly less than 200,000 daltons for the antigen(s) responsible for the formation of neutralizing antibodies.

Viral structural polypeptides have recently been studied in more detail with the use of two techniques. First, degradation in SDS and analysis by acrylamide gel electrophoresis in SDS has shown the virus to be composed of at least 17 polypeptides of different molecular weights

(Holowczak and Joklik, 1967). Second, the controlled degradation of the virus by detergents has helped in the localization and nature of certain structural components (Easterbrook, 1966). More recently, better techniques of gel electrophoresis have resolved more than 30 polypeptides, ranging in molecular weights from 8,000 to 250,000 daltons (Sarov and Joklik, 1972a; Obijeski et al., 1973).

Chemical analysis of these polypeptides has shown that at least one and possibly two were glycopeptides and were located in the external or outer region of the virus (envelope and surface protein) (Holowczak, 1970; Garon and Moss, 1971; Sarov and Joklik, 1972a). The use of ^{32}P has indicated that at least six polypeptides of low molecular weight (less than 50,000 daltons), were phosphorylated most probably on a serine or threonine residue (Downer et al. 1973).

Some of the 30 viral polypeptides have very specific functions other than structural. So far, six of them possessing enzymatic activities, have been associated with the virus core. These are a DNA-dependant RNA polymerase (Kates and MacAuslan, 1967a; Munyon et al., 1967), two deoxyribonucleases, a neutral endonuclease and an acidic exonuclease (Pogo and Dales, 1969), one acidic and one alkaline nucleotide phosphohydrolase (Gold and Dales, 1968; Munyon et al. 1968), and a protein kinase (Paoletti and Moss, 1972; Downer et al., 1973).

The DNA-dependant RNA polymerase has been implicated in the formation of the enzymes responsible for the final uncoating of the viral genome (Joklik, 1966; Woodson, 1968). Walen (1971) found that contrary to previous reports, the host DNA was degraded to a certain extent and suggested that the viral DNases were responsible for this degradation. Recently, a new role has been attributed to the DNases associated with the viral core (Pogo and Dales, 1973). During infection, the DNases (or at least one of the two), invade the cell nucleus and inhibit host DNA replication. Definitive proof of this role is still lacking, but is important because if true, it would be the first time that an enzyme associated with the invading particles would be involved in the inactivation of a specific host-cell function.

It has been suggested that the phosphohydrolases have a regulatory role. Since the viral DNA-dependant RNA polymerase requires an ATP regenerating system to be fully active, it was thought that this system, by limiting the amount of ATP, could somehow regulate the level of the RNA polymerase activity or some unknown function (Gold and Dales 1968).

Recently, a poly A polymerase has been detected in the core of vaccinia virus (Brown et al., 1973). This polymerase is different from the DNA-dependant RNA polymerase, as judged by the pH activity profile, inhibition studies, heat stability and the fact that it can utilize only ATP as a substrate.

BIOGENESIS OF VACCINIA VIRUS

The biogenesis of vaccinia virus can be divided into four parts: entry, biosynthesis of virus-specified proteins, morphogenesis and release of the virus particles.

The entry of the invading particles has been well documented biochemically and by electron microscopy (Dales and Siminovitch, 1961; Dales, 1963; Joklik, 1964a, 1964b; Sarov and Joklik, 1972b; Holowczak, 1972). The virus is taken into the cell by viropexis. While still inside a vacuole, there is removal of the external envelope followed by a lag period during which there is active and essential synthesis of RNA and protein, directed or originated from the partially digested viral particles (Kates, 1967b; Woodson, 1968). After this lag period, which is essential for the synthesis of the "uncoating" protein (Joklik, 1966) . the lateral bodies are pushed away from the central core and digested (Dales, 1973). At the same time, most of the enzyme activities associated with the core are increased, suggesting that possibly one of the roles of the lateral bodies is to mask these activities as some kind of crude regulatory mechanism (Pogo and Dales, 1969).

Finally, the cores without the lateral bodies and the invading vacuole, are digested with subsequent release of the parental DNA in the cytoplasm of the host cell. The DNA will be rapidly replicated in what are called "cytoplasmic factories" (Cairns, 1960). These "cytoplasmic factories" consist of the material necessary for the production, regulation and assembly of the final viral particle.

Before and after replication of the parental genome, there is sequential protein synthesis (Salzman and Sebring, 1967; Moss and Salzman, 1968). This synthesis is subjected to a switch-on and switch-off mechanism giving rise to early proteins whose synthesis is directed by the parental genome and late proteins whose synthesis is directed by the progeny DNA (Holowczak and Joklik, 1967b; Wilcox and Cohen, 1967).

The early proteins are responsible for the genome replication, regulatory mechanisms and some are also structural proteins (Holowczak and Joklik, 1967b). Recently, a classification unique to poxviruses has been proposed by Esteban and Metz (1973). The early proteins can be divided into two classes: a) molecules synthesized under the direction of the uncoated parental DNA and b) molecules synthesized even before the parental DNA is uncoated. The latter class would use the DNA dependant RNA polymerase attached to the viral core. Their functions (early proteins b) are still unknown but might involve a role in early cytopathic effect (Bablanian, 1970) and in the final uncoating of the viral core since if the RNA synthesis is stopped by inhibitors, the cores are not digested (Joklik, 1964b; Kates and MacAuslan, 1967b). The studies, utilizing cytoplasmic extracts of infected cells have shown that early proteins were of low molecular weights (50,000 to 100,000 daltons), while most late proteins of which the majority are regulatory and structural proteins, had a molecular weight of 200,000 daltons or greater (Wilcox and Cohen, 1966).

So far, all the enzymes associated with the viral core have been classified as late proteins (Nagayama et al., 1970).

The morphogenesis of vaccinia virus starts shortly after DNA replication. The first morphological components to appear in the cytoplasm, are small arc-like fragments consisting of a trilaminar membrane covered with spicules (Dales and Siminovitch, 1961; Dales and Mosback, 1968). These small membrane fragments elongate and eventually enclose each immature particle in a spherical envelope. At that stage of development, the immature forms are substantially larger than the mature forms and no internal details are seen, except for a well dispersed electron dense matrix.

The matrix condenses in the center of the immature forms where it is then coated by the core proteins and the lateral bodies (Dales and Mosbach, 1968). During this sequence of events, the particles are reduced in size and acquire their typical brick-shaped morphology.

Virus maturation is well regulated. The studies of Nagayama et al. (1970) using the inhibitor rifampicin, have shown that the late functions performed by the late proteins are used extensively for the maturation process and its regulation, while the early functions performed by the early proteins are directed toward the formation of structural components necessary for the morphogenesis of the virus. One of the major components of the virus core is synthesized as a late protein with a molecular weight of 125,000 daltons. Just before or shortly after assembly into the maturing viral particle, this component is digested to a molecular weight of 76,000 daltons (Katz and Moss, 1970a; 1970b). Recently, pulse chase experiments showed that three or more viral induced polypeptides were also formed by cleavage of the larger precursors but only one of them was eventually incorporated into the mature virus (Pennington, 1973).

The completed virus is then released from the cell by reverse viropexis. There is some controversy as to whether the virus acquires a membrane or an envelope during the process of viropexis. Dales (1963), did not see any association of that kind but Appleyard et al. (1971), using a rabbitpox virus, demonstrated electronmicroscopically and biochemically the presence of an envelope covering the virus. Electron microscopic examination of extracellular virions showed clearly this envelope which could be destroyed by brief ultra-violet treatment. Antiserum produced against this extracellular virus could neutralize intracellular virions which are devoid of envelope but the reverse was not found.

So far, it is not known if this envelope is viral coded or if it contains host cell protein. The only evidence suggesting the presence of host cell material associated with vaccinia virus comes from the work of Rybakova et al. (1969a; 1969b). These authors were able to neutralize vaccinia virus by using antiserum produced against host cell membrane antigens. Unfortunately, they did not state whether they had used intra- or extracellular viruses, for their neutralization experiments.

ISOLATION AND LOCALIZATION OF VACCINIA VIRUS STRUCTURAL COMPONENTS

Because of the extreme resistance of vaccinia virus to various treatments, the viral structural components have not been properly characterized (Marquardt et al., 1965; Cohen and Wilcox, 1966; Marquardt et al., 1969).

The antigens called NP (for nucleoprotein) and LS (because the antigen contains a heat-labile and a heat-stable antigenic determinant) were found to be composed of many proteins (Smadel, 1942). Marquardt et al. (1969), partially purified a protein with properties similar to the LS antigen. This protein had a molecular weight of 90,000 daltons and amino acid composition showed large amounts of glutamic and aspartic acid to be present. These authors could detect three immunological specificities associated with the antigen: two were heat-labile and one was heat-stable.

Becker and Stephen (1971), using soluble viral proteins, partially purified two structural antigens, but which were analysed only for their heat and pH stability. The acid nucleotide phosphohydrolase was extracted from purified vaccinia virus by sodium deoxycholate and purified by isoelectric focusing (Paoletti and Munyon, 1971). This enzyme had partial immunological identity with the LS antigen and could elicit the production of neutralizing antibodies. If this observation is ever confirmed, it would be the first time that an enzyme associated with a virus be implicated as an antigen involved in the formation of virus neutralizing antibodies.

The use of an anionic detergent (SDS) and a technique to obtain cores by treatment with NP-40 and 2-mercaptoethanol (Easterbrook, 1966),has enabled Joklik and Holowczak (1967a) to give a crude localization of 17 polypeptides composing the virus. Three of the polypeptides were associated with the core while the rest were present in the lateral bodies, the external envelope and the surface protein coat.

More recently, improved electrophoresis techniques have shown that the virus contained more than 30 polypeptides with molecular weights ranging from 8,000 to 250,000 daltons. Furthermore, Sarov and Joklik, (1972a) using various special staining techniques and autoradiographic methods, found the cores and the lateral bodies to be composed of 17 polypeptides leaving a total of 13 polypeptides present in the external envelope and in the protein coat (the latter two components are called the outer region or the external layer of the virus).

The use of two techniques, (a) enzymatic iodonation of the surface proteins using lactoperoxidase, hydrogen peroxide and ^{125} I and (b) treatment of the virus particles with isothiocyanate, has indicated that five distincts polypeptides were situated on the viral surface (Sarov and Joklik, 1972a). The major components of the outer region of the virus, the VP-6 family of polypeptides, were not found on the virus surface. This is in contradiction with the recent findings of Katz and Margalith (1973) who, using the same technique of enzymatic iodination as that and by Sarov and Joklik, found that among the major components of the virus surface was the VP-6 group of polypeptides.

It is obvious that the knowledge of the structure and of the assembly of the viral components is incomplete and more work is needed. The present study was undertaken to learn more about the structure and the composition of vaccinia virus. The viral structure and composition were studied by examining detergent extracts from the viral particles by SDS gel electrophoresis and by examining the extracted viral particles themselves, by SDS gel electrophoresis and by electron microscopy. An antiserum was obtained against a purified structural antigen extracted by the detergent NP-40. This serum was used to locate in the viral particles, the extracted viral antigen by the technique of immunoelectron microscopy.

Since the time this work was completed and the thesis typed, there have been some references which are relevant to our work.

Some of the enzymes associated with the core of the virons have been partially purified and characterized to some extent. Kleiman and Moss (1975) using cellulose chromatography have purified the protein kinase of the virus. It has a molecular weight of 62,000 daltons and needs protamine as an activator. Furthermore, two protein acceptors associated with the outer layer of the virus were also purified and found to have molecular weights of 38,500 and 11.700 daltons.

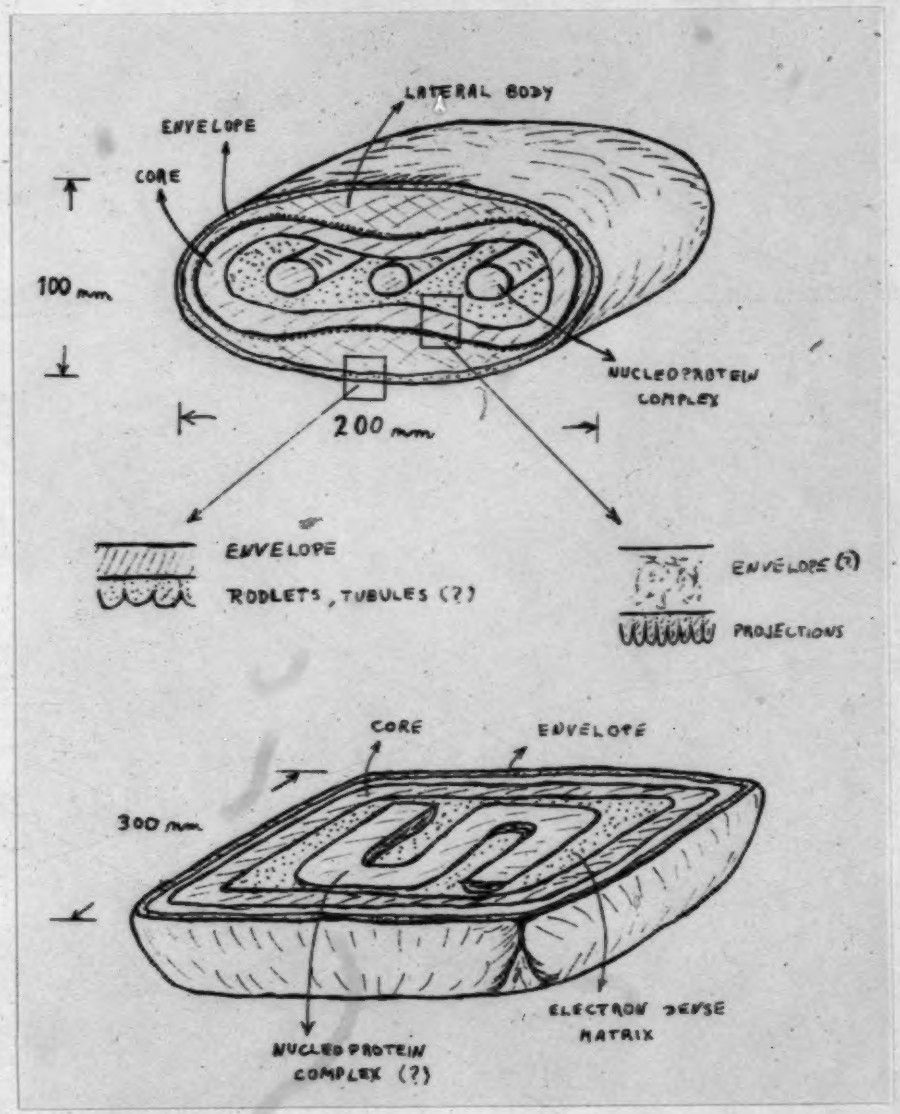
Concerning the association of viral polypeptides with the DNA of the virus, - Pogo et al. (1975) and Lanzer and Holowczak (1975) have found that polypeptides of 11,000-12,000 and 34,500 daltons (possibly VP-6) were associated with the viral DNA.

The use of SDS discontinuous gradient polyacrylamide gel electrophoresis has permitted a much better resolution of the polypeptides composing the virus. McCrae and Szilagyi (1975) have found a total of 48 distinct bands using a 5-15% gradient of polyacrylamide. Their pattern of the bands corresponded very well with the previous work of Sarov and Joklik (1972a) but with an increased resolution of the various groups of polypeptides.

FIGURE I

SCHEMATIC REPRESENTATION OF VACCINIA VIRUS

This schematic representation of the structure of vaccinia virus was made from information and values obtained from published work and from personal observation.


LATERAL BODY
ENVELOPE
CORE
100 mm
NUCLEOPROTEIN COMPLEX
200 mm
ENVELOPE
RODLETS, TUBULES (?)
ENVELOPE (?)
PROJECTIONS
CORE
ENVELOPE
300 mm
ELECTRON DENSE MATRIX
NUCLEOPROTEIN COMPLEX (?)

MATERIALS AND METHODS

VIRUS

The vaccinia virus strain used was the "Wyeth-calf adapted" obtained from the American Type Culture Collection (ATCC). It was passed 14 times in a monkey kidney cell line (LLC-MK2) and stored at -70°C.

CELL CULTURE

The monkey kidney cell line (LLC-MK2) was obtained from the American Type Culture Collection. It was used for all virus assays and virus production. These cells were grown in Roux and roller bottles.

CELL COUNTING

Cells were dispersed and diluted in PBS pH 7.2 and stained with Trypan Blue (0.5% in water) for viable count or with crystal violet (0.1% in 0.1M citric acid) for total count. The stained cells were counted using a hemocytometer (Phillips and Terryberry, 1957).

TISSUE CULTURE MEDIA

Powdered minimal essential medium (MEM) was obtained from Grand Island Biological Company GIBCO, Grand Island , N.Y. and was used as growth medium in all the experiments. It was supplemented by 10% calf serum (prepared in this laboratory), 100 International Units per ml of penicillin G and 100 µg per ml of streptomycin sulfate. The pH of the growth medium was adjuted to 7.6 by the addition of sodium bicarbonate.

Hanks balanced salt solution (HBSS) (Hanks and Wallace 1949) containing phenol red as a pH indicator was used for washing cells and also as a diluent for viral assays.

The MEM 2X used for the viral plaque assay was prepared by dissolving the powdered medium in half the amount of water normally required.

PLAQUE ASSAY

The plaque assay system used was the double overlay technique (McClain et al., 1967). Cells were grown in 3 ounce pharmaceutical bottles until confluent. At that time, they were examined to insure that the cell sheet was intact. The cells were washed once with HBSS and 0.3 ml of the various viral dilutions made in HBSS were inoculated. The incubation period was 1 h at 37°C with gentle rocking of the bottles every 15 min to ensure an even distribution of plaques.

The cell sheet was then carefully covered with a 10 ml overlay of the following composition: 1.5% Noble Agar and 5% calf serum in MEM. After solidification of the overlay, the bottles were inverted and incubated at 37°C. After 2 days in incubation, a second 10 ml overlay was added. The composition was the same as the first one, except for the addition of neutral red to a final concentration of 0.01%. Incubation was carried on in the dark at 37°C, and the plaques were counted after 24 and 48 h.

NEUTRALIZATION ASSAY

The assay system used was the 50% plaque reduction test. Essentially, the same plaque assay as described above was carried out using as an inoculum, a viral suspension treated with antiserum.

To a viral suspension containing 200 plaque forming units (PFU)/0.3 ml was added an equal volume of the antiserum to be tested. The serum had previously been inactivated at 56°C for 30 min and diluted in PBS. The reaction mixture was well shaken and incubated at 37°C for 1 h. Then 0.3 ml of this mixture was used for assay of the residual infectivity by the plaque assay method.

Controls were made using HBSS instead of virus and a sample of serum obtained from the same rabbits used for the production of the antiserum, but taken before immunization. The titer of the serum was expressed as the reciprocal of the dilution that would reduce by 50% the number of plaques obtained in the control.

GROWTH CURVE

A growth curve was determined for this particular strain of virus, since it had not been passed before on LLC-MK2.

The cells grown in 3 ounce bottles were washed with HBSS and inoculated with 0.3 ml of a viral suspension, so as to give a final moi of 15 PFU/cell. The adsorption period was 90 min at 37°C with gentle rocking of the bottles every 15 min. After the adsorption period, the inoculum was removed, the cell sheet was washed with 5 ml of HBSS and 10 ml of fresh growth medium was added. The residual moi found after measuring the number of PFU's in the inoculum and washing fluid, was measured to be 13.5 PFU/cell. Every 2 h after infection, 2 sample bottles were taken and their supernatants were centrifuged to sediment floating cells so that the total number of extra-cellular PFU could be counted. The remaining cell sheet was immediatly washed, frozen and stored at -70°C. Shortly after, it was thawed, and the cells were scraped off the glass, frozen and thawed 5 times. The broken cells were centrifuged at 1500 rpm for 10 min and the supernatant was used to measure the total number of intracellular PFU's. All the counts were done by the plaque assay technique and expressed as PFU/cell. The average number of cells in a 3 ounce bottle was an average count of 8 bottles pooled and counted shortly before inoculation.

PROTEIN DETERMINATION

1) Lowry method

Protein concentration was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard (Salzman and Sebring, 1967).

2) Absorbance

Appropriate dilutions of the material were made in 0.1N NaOH and incubated for 30 min at room temperature. Then the ratio of absorbance at 280 nm over 260 nm was measured and protein concentration determined as recommended by Layne (1957).

3) Protein concentration of viral suspension

Vaccinia virus purified twice by sucrose density gradient centrifugation was used as a standard to determine the relationship between absorbance at 260 nm and protein concentration determined by the Lowry method. It was found that 1 absorbance unit at 260 nm corresponded to 81 µg of viral protein per ml of a well dispersed suspension of vaccinia virus.

PREPARATION OF VACCINIA VIRUS

1) Production of stock virus

Twenty five Roux bottles were inoculated with 5 ml of 15th passage of virus strain titrating at 5×10^7 PFU/ml. The virus was allowed to adsorb for 90 min at 37°C. After the adsorption period, the inoculum was discarded and 100 ml of fresh MEM was added.

Following two days of incubation, when the entire cell sheet showed cytopathic effect the cells were then detached from the glass, collected by centrifugation at 1500 rpm for 10 min, and the pellet resuspended in 100 ml of MEM. The cells were disrupted by 5 cycles of freezing and thawing. The cell debris was pelleted by centrifugation at 5000xg for 30 min and the clear supernatant was distributed in small aliquots and stored at -70^{o}C.

The titer of the stock virus was obtained by $2x10^{8}$ PFU/ml and decreased only one log unit over a period of 4 years.

2) Production of large quantities of virus

To obtain large quantities of virus, the roller bottle system was employed. A sample of the stock virus was used to inoculate a roller bottle at a moi of 0.1 PFU/ml. After incubation for 48 h, the cells were collected by centrifugation, chilled in an ice water bath and sonicated a total of 60 seconds (4x15s) with a Biosonik III sonicator (Bronwill Scientific, Rochester, N.Y.). This extract was used as the inoculum for 10 roller bottles. This system yielded large quantities of virus. The infection of 30 roller bottles could produce a crude extract containing 10^{12} PFU's. This amount was found to be sufficient for the purification of viral proteins obtained from purified virions.

Assuming that 10^9 PFU's represent approximately 80 µg of viral protein (Pogo and Dales, 1969), 80 mg of unpurified vaccinia virus could be produced by 30 roller bottles.

3) Preparation of virus for purification

Vaccinia virus was purified by centrifugation either in cesium chloride (Planterose et al., 1962) or in sucrose (Joklik, 1962b; Zwartow et al., 1962).

Roller bottles (12 x 20 cm) were seeded by adding 100 ml of MEM containing $1\text{-}2\times10^5$ cells/ml or by adding the cell content of a whole Roux bottle to 100 ml of MEM. The bottles were rolled at a speed of one half turn per minute at 37°C. Confluence was reached after 3-5 days; at that time the number of cells per bottle was usually $1\text{-}3\times10^8$.

The cells were washed once with HBSS and infected by adding 10 ml of inoculum to give a final moi of 1-2 PFU/cell. After an adsorption period of 90 min at 37°C, 90 ml of fresh growth medium were added and the incubation continued for 48 h. At that time, the cell sheet showed complete cytopathic effect.

The cells were detached from the glass and collected by centrifugation at 1500 rpm for 15 min. The cell pellet was resuspended in a small volume of PBS (usually 0.5 ml per roller bottle), and homogenized in a glass tube tissue homogenizer using a Teflon pestle, until 90-95% of the cells were broken. After homogenization, the preparation was adjusted to pH 8.2 with 0.1N NaOH and sonicated

(3x15 s) to reduce adsorption of virus to cell debris. This crude extract was centrifuged at 3000xg for 20 min to remove large debris. The milky white supernatant was used for further purification.

When the supernatant was in excess of 10 ml, the viruses were pelleted at 40,000xg for 90 min. The supernatant of this centrifugation was kept as a "soluble antigen fraction". The viral pellet was resuspended in 5 ml of buffer (Tris-HCl, 0.001M, pH 8.2), by agitation and light sonication (2x10 s).

Before homogenization, the cell suspension was cooled to 4°C and all the subsequent operations were performed at that temperature to minimize viral degradation.

All the purification steps were carried out in the same day and at no time was this semi-purified suspension ever stored.

PURIFICATION OF VACCINIA VIRUS

1) Purification by cesium chloride gradient centrifugation

The crude viral suspension (adjusted to pH 8.2), was layered on a 20-40% (w/w) linear gradient of CsCl in 0.001M Tris-HCl buffer, pH 8.2. The gradient was centrifuged at 75,000xg for 3 h (SW39L rotor, Beckman Inst., Palo Alto, Calif.). The viral band was removed from the top of the tube using a syringe. The viral suspension was dialysed overnight at 4°C against PBS and stored at -70°C.

2) Purification by sucrose gradient centrifugation

For most studies virus was purified by means of centrifugation in a sucrose density gradient.

The crude viral suspension (adjusted to pH 8.2), usually 2.0-2.5 ml, was gently layered on a pre-cooled (4°C) 20-40% linear gradient of sucrose in 0.001M, Tris-HCl, pH 8.2. The volume of the gradient was 30 ml so that at all times, the viral suspension was less than 10% of the volume of the gradient.

The gradient was centrifuged at 25,000xg for 45 min in a HB-4 swinging bucket rotor (I. Sorvall, Norwalk, Conn.). The viral band was collected from the top of the tube with a syringe and dialysed overnight at 4°C against PBS. The dialysed suspension was stored at -70°C. When a second centrifugation in sucrose was used, the dialysed preparation was pelleted by centrifugation at 40,000xg for 90 min. The pellet was resuspended in 2.5 ml of Tris buffer by sonication (2x10 s) and layered onto a fresh gradient.

The purity of the viral preparation was checked by electron microscopy using negative contrast staining and by absorbance ratio at 260 nm and 280 nm (Pfau and McCrea, 1963). In some experiments, the preparations were screened for the presence of viral and host cell soluble antigens by double gel diffusion, using appropriate antisera.

PREPARATION OF IMMUNE REAGENTS

1) Preparation of viral antiserum

Antisera directed against both active and U.V. inactivated virus were used throughout these experiments.

Vaccinia virus recovered after two successive centrifugations in a sucrose gradient was used as active virus. The injection schedule was similar to that of Lennette and Schmidt (1969). Three month old male San Juan rabbits were inoculated intra-dermally with 1 ml of the viral suspension (5 sites of injection on the back). Pustules developed at every site of injection in 2-3 days, showing that this dermal strain was still virulent, even after more than 15 passages in tissue culture.

Four weeks after the intra-dermal injection, the rabbits received 1 ml of the same viral suspension, intra-muscularly. This intra-muscular injection was repeated every week for three more weeks. The total amount of viral protein injected into each rabbit was 1.25 mg (250 μg each time). Ten days after the last injection, the animals were bled and the sera collected, filtered and stored at -20°C.

The antiserum produced against U.V. inactivated virus was obtained from Dr. S. Shahrabadi. The procedure used to obtain the antiserum has been described (Shahrabadi, 1972).

2) Preparation of host cell antiserum

In order to detect the presence of host cell soluble proteins, an antiserum was prepared against a

LLC-MK2 cell fraction prepared as follows. Cells were collected from the roller bottles and treated as the virus infected cells. The high speed centrifugation (40,000xg for 90 min) supernatant was the material injected into the rabbits. The total amount of protein injected into each rabbit was 23.5 mg. Antiserum was prepared as described for virus preparation.

When the antiserum so obtained was analysed by microimmunodiffusion and ring test, a 1/100 dilution still gave a precipitin reaction when tested against host cell soluble antigens.

3) Preparation of antiserum against complex E

Antiserum was produced against a viral structural component called complex E (see page)

Rabbits were injected with a total of 500µg of viral complex E. The soluble antigen was emulsified with Freund's complete adjuvant for the first injection which was given in the toe pads of the animals. The subsequent injections were given in the toe pads and sub-scapulary region. The antigen solutions were emulsified in Freund's incomplete adjuvant 24 h before injection. Subsequent injections were given 5 and 8 weeks later.

Two and three weeks after the last injection, the rabbits were bled and sera sterilized by filtration and sodium azide added as a preservative. Half the sera were frozen at -20°C, the other half kept at 4°C.

4) Purification of gamma-globulin

The procedure used to obtain immunoglobulin from goat serum was that of Breese and Hsu (1971). Goat serum (anti-rabbit gamma-globulin) was precipitated 5 times by sodium sulfate at 20% saturation for 3h at room temperature. The final precipitate was dissolved in PBS and dialysed overnight at 4°C against PBS. The final solution had a protein concentration of about 35 mg/ml. Immunoelectrophoresis analysis of this solution indicated that the material obtained by sodium sulfate precipitation had a precipitation pattern characteristic of gamma-globulin.

The goat globulin anti-rabbit globulin fraction was stored at -20°C with 0.01% sodium merthiolate added as a preservative.

DETERGENT EXTRACTION

Purified viruses were extracted with various types of detergents. The following procedure was used for all kinds of detergents (Nonidet P-40, Brij 35, Brij 58, Tween 80, Saponin, Triton X-100, Sodium deoxycholate and Cetyl Trimethyl ammonium bromide).

The viral suspension at a concentration of 1 mg of viral protein per ml, was dialysed overnight against 10mM phosphate buffer, pH 7.4. Detergents were added until a final concentration of 0.5% (w/w) was reached. At all times during the different extractions, the ratio (w/w) of detergent to viral protein was maintained at 5:1. In other words, the solutions had the same amount of detergent (in mg) per mg of viral protein. At the end of the incubation period, a few drops of the suspension were immediately put on electron microscopic grids, stained negatively with PTA and examined with the electron microscope.

The rest of the suspension was centrifuged at 40,000xg for 90 min to pellet the residual viruses. The supernatant was recovered and either dialysed against PBS or stored at -20°C. The extract following treatment with non-ionic detergents were dialysed but those following treatment with ionic detergents were not dialysed. Rather, they were used immediatly or stored, since dialysis resulted in extensive precipitation of the extracted material.

The extracted viral pellets were resuspended, washed in PBS and stored at -20°C for further analysis. Since after extraction the residual viral structures might be fragile, the viral pellets were gently resuspended after centrifugation.

CHEMICAL EXTRACTION

Purified virus was resuspended to a concentration of 1 mg of viral protein per ml. This suspension was brought to a final concentration of 6N guanidine hydrochloride or 8M urea by adding the denaturing agents as crystals, directly into the suspension. These mixtures were incubated for 3 h at 37°C on a small rotatory shaker and subsequently treated as in "detergent extraction".

An alkaline treatment in 0.05M glycine buffer, pH 10.5 was also carried out on the virus resuspended at 1 mg/ml. After incubation, the suspension was treated as in "detergent extraction".

GEL CHROMATOGRAPHY

The system described in "Results" was prepared according to the manufacturer's methods and specifications. The gel chromatography operations were carried in the cold (4°C). The technique of upward flow chromatography was used. The fractions were collected by the drop counting technique using an Ultrorac fraction collector (LKB, Sweden).

IMMUNODIFFUSION

Samples to be analysed were dialysed overnight against PBS at 4°C. Samples and antisera were diluted in PBS. The microimmunodiffusion technique of Crowle (1958) modified by Grasset (1967) was used. The plastic templates had 6 peripheral wells 3 mm in diameter. A seventh central well of identical diameter was 4 mm from all other wells. The well cavities were filled with 25 µl of antiserum or antigen solution. The diffusion in a humid chamber at room temperature or at 4°C, was through an agar layer 1 mm thick, composed of 0.75% Agarose, 0.85% NaCl and 0.02% merthiolate in 0.1M phosphate buffer pH 7.2. In some cases, microimmunodiffusion plates already prepared were used (IDF II, Cordis Laboratories, Florida). The distance of diffusion was 3.2 mm and each well received 25 µl of solution. The composition of the agar layer (thickness of 1 mm) was: 1% Agarose and 0.1% sodium azide in PBS pH 7.2.

The plates were examined daily under indirect lighting for the presence of immunoprecipitate. Photographs were taken with indirect lighting after 3-5 days of incubation, using Kodak Plus-X Pan film.

IMMUNOELECTROPHORESIS

Immunoelectrophoresis was carried out in the following manner: acid washed plates (5x12 cm) previously coated with a 0.2% Noble Agar, were covered with 20 ml of melted agar to give a thickness of about 4 mm. The agar solution was prepared by mixing a solution of 2% Noble Agar in water with an equal volume of warmed (45°C) 0.04M sodium barbital-acetate buffer pH 8.4 containing 0.4% sodium azide. After hardening, wells of 1.5 mm in diameter and troughs 1.5mm wide were cut through the agar. The distance between wells and troughs was 5 mm. The wells were filled with about 15 µl of antigen solution previously dialysed against PBS.

The plates were electrophoresed at a potential of 5 volts/cm of agar between the wicks for 1 h. After electrophoresis, the agar from the troughs was removed and the troughs filled with the proper antiserum. The plates were incubated in a humid chamber at room temperature or at 4°C. They were examined daily for the presence of precipitin bands under indirect lighting and photographs were taken as for immunodiffusion.

ANALYTICAL ULTRACENTRIFUGATION

The material to be analysed by sedimentation velocity was diluted with 0.01M phosphate buffer pH 7.2 to a concentration of 1-2 mg/ml. The centrifugations were carried out at 4° C using a Model E Analytical Ultracentrifuge (Beckman Instruments Inc., Palo Alto, Calif.) and a rotor type D at 60,000 rpm. Photographs were taken periodically after a boundary was first seen on the Schlieren optical system, i.e. after the first sign of appearance of a peak. Photography was continued until the peak disappeared.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Acrylamide and bis-acrylamide were recrystallized before use, as recommended by Loening (1967). Acrylamide was recrystallized from chloroform while N,N',-methylenebis-acrylamide was recrystallized from acetone. All the solutions used for gel electrophoresis were kept in dark bottles and made fresh every 6 months except for ammonium persulfate which was made fresh weekly.

1) Discontinuous pH disc gel electrophoresis

Discontinuous pH disc gel electrophoresis was performed essentially according to Davies (1964).

The small pore or separating gel contained 7% acrylamide and 0.184% bis-acrylamide. Ammonium persulfate was used to provide the free radicals necessary to promote polymerization. The stacking gel or spacer gel had 2.5%

acrylamide and 0.625% bis-acrylamide. Polymerization was achieved with a fluorescent light to provide free radicals from riboflavin.

The sample gel had a composition identical to the stacking gel except for the addition of 5% sucrose and 0.005% bromephenol blue as a tracer dye.

The sample contained between 5 and 200 µg of protein. For very dilute samples, the sample gel was omitted and the sample containing 5% sucrose was layered directly on top of the stacking gel.

The inside diameter of the glass tube was 5 mm and the lengths of the various gels were 7 cm, 1.5 cm, and 1.5 cm for the resolving, stacking and sample gels, respectively. The gels were electrophoresed at 2 mamp/gel for 3 h or until the tracer dye reached 0.5-1 cm from the bottom of the tube.

The gels were removed from the glass tubes and fixed overnight in 20-30 volumes of 10% TCA. Then they were washed with water and stained for 3 h in 20 volumes of a 0.25% solution of Coomassie Brilliant Blue R-250 in water. They were destained by 3 washes in 7% acetic acid and stored in the same solution.

2) Neutral SDS gel electrophoresis

Neutral SDS gel electrophoresis was performed essentially as described by Maizel (1971).

The sample containing 5-200 µg of protein in a volume of 50 µl was dissolved in a 50 µl solution containing

2% SDS, 0.01 M dithiothreitol and 1M urea, in 0.001M phosphate buffer pH 7.2. The mixture (100 μl) was incubated at 37°C for 3 h and then put in a boiling water bath for 2 min. The sample was then made up to 10% sucrose and 0.005% bromphenol blue.

This sample was carefully layered on top of a gel containing 10% acrylamide, 0.27% bis-acrylamide and 0.1% SDS in 0.1M phosphate buffer pH 7.2. The gels (0.5x10 cm) were run in the presence of 0.1% SDS in 0.1M phosphate buffer pH 7.2. The electrophoresis was performed at 1 volt per cm of gel for 30-45 min. The voltage was then increased to 5 volt/cm for about 8 h or until the tracer dye reached 0.5-1 cm from the bottom of the tube.

The gels were removed from the glass tubes and fixed overnight in 20 volumes of a 20% sulfosalicylic acid solution. The gels were then washed with tap water and stained for 4 h in a solution containing 0.25% Coomassie Brilliant Blue R-250 in water. The destaining was done by three washings in 7% acetic acid and the gels were stored in the same solution.

CHARACTERIZATION OF THE POLYPEPTIDE BANDS

1) Protein staining

Various types of staining were utilized in an attempt to identify the nature of some of the viral structural polypeptides directly on the gels.

Coomassie Brilliant Blue was preferred for protein staining since it had a higher sensitivity than most other stains (Maurer, 1971).

2) Lipid and carbohydrate staining

Staining of gels for the identification of glycopeptides was done using the PAS technique (Periodic Acid Schiff) as described in Maurer (1971). The gels were fixed overnight in 10% TCA and put in a solution containing 3% periodic acid in 3% acetic acid for 1 h. They were then washed in five changes of distilled water for 1 h. After that time, they were immersed in Schiff's reagent for 1 h and then transferred to a solution containing 1% sodium metabisulfate in water for 1 h. They were stored in the same solution.

Staining for lipopeptides was done essentially as described by Pratt et al. (1969). The gels were fixed in TCA and stained overnight in the following solution: 500 mg of Sudan Black B was dissolved in 20ml of acetone; to that was added 15 ml of glacial acetic acid and 80 ml of distilled water. The solution was stirred for 30 min and centrifuged or filtered by suction. The prepared stain was stable for only 2 days. The gels were destained by repeated washes in the same solvent system but without the Sudant Black B.

3) Densitometer tracing of the stained gels

The gels were scanned using a Gilford scanning attachment coupled to a Gilford Spectrophotometer 240. The slit width was 0.5 mm and the scanning speed was 2 cm/min. The patterns were traced by a Photovolt Varicord

43 recorder (Photovolt Corporation, N.Y.) at a speed of 2 inches per minute. All recordings were made to obtain maximum impulse of the recorder, so that unless mentioned the height of the peaks on the densitometer tracings cannot be compared with each other. All the densitometer tracings will have then an arbitrary scale for relative intensity (absorbance). For Coomassie Brilliant Blue stained gels, the scanning was at 585 nm; for PAS stained gels, 545 nm and for Sudan Black B stained gels, 590 nm.

4) Molecular weight determination

The determination of the apparent molecular weight for the various polypeptides was done as described by Weber and Osborn (1969). A standard curve was made for gels containing 10% acrylamide and 0.2% bis-acrylamide by using proteins of various molecular weights (gamma-globulin, glucose-6-phosphate dehydrogenase, bovine serum albumin, trypsin and cytochrome c).

ELECTRON MICROSCOPIC TECHNIQUES

1) Negative contrast staining

Two methods were used when viral preparations were examined by negative contrast staining. In the first method, the sample was mixed directly with an equal amount of 3% sodium phosphotungstate (pH 7.0), and a drop of the mixture was deposited on a 200 mesh electron microscope grid coated with Formvar (0.25% in 1-2 dichloroethane) and strengthened with carbon. The drop was left on the grid for 60 s, then drained. After drying, the grid was examined using a Phillips 200 electron microscope at 60 Kv.

When the samples contained high concentrations of salts or detergents, they were put directly onto the grids. After 60 s the drop was drained and the grid was allowed to dry. It was then washed with a stream of distilled water for 15-30 s. After drying, the grid was stained with 3% sodium phosphotungstate and examined.

2) Ultramicrotomy

All embedded materials were sectioned by a diamond knife (E.I. Dupont de Nemours and Company, Wilmington, Del.) fitted to a Porter-Blum MT-2 Ultramicrotome (I. Sorvall, Norwalk, Conn.). Sections embedded in Epon 812 were stained with 2% uranyl acetate in methanol for 10 min and lead citrate (Reynolds, 1963) for 5 min.

Material embedded in glycol methacrylate had to be warmed to room temperature before being sectioned. Because this polymer is water soluble, the sections were floated in the water boat for 10 min to allow then to swell before being picked up. The sections were stained in 0.8% uranyl acetate in water for 1 h and with lead citrate for 2-3 min.

3) Counting of viral particles

The following technique modified from Monroe and Brandt (1970) was used to obtain an estimate of the number of viral particles.

Standardized latex particles (Dow Chemical Company, Midland, Michigan) (264 nm in diameter) at a concentration of 3.46×10^9 particles/ml were mixed with equal volumes of a properly diluted viral suspension (previously sonicated) and a solution containing 3% sodium phosphotungstate and 0.25% bovine serum albumin at pH 7.0. Normally, 10 µl of each solution was mixed and 5 µl of the mixture was deposited on a 400 mesh electron microscopic grid. After 1 min the excess liquid was removed and the grid allowed to dry. The grid was examined at low mignification to ensure the absence of large clumps of viruses or latex particles. If clumps were found, a new preparation was made. When 400 mesh grids were utilized, counts for latex and viral particles were obtained for at least 6 squares. Knowing the original concentration of latex particles, the number of viral particles per ml could be estimated.

FIXATION OF VACCINIA VIRUS FOR ELECTRON MICROSCOPY

The method of fixation parallels the one recommended by Glauert (1965). The virus was washed and resuspended in a solution containing 3% glutaraldehyde in Sorensen's (0.1M phosphate) buffer pH 7.2 containing traces of $CaCl_2$, for 3 h at 4°C. After fixation, the virus was washed 3 times in Sorensen buffer containing 10% sucrose. The virus was left in the last wash overnight. The virus was again pelleted and resuspended in a solution containing 2% osmium tetroxide in 0.1M phosphate buffer pH 7.2, for 2 h at 4°C.

After the second fixation, the virus was repelleted in a micro-centrifuge tube. The white pellet was embedded in 2% agar as recommended by Kellenberger et al. (1958) and cut into 1 mm cubes. The cubes were dehydrated in alcohol at 4°C, rinsed in propylene oxide for 30 min and left overnight in a 1:1 mixture of propylene oxide and Epon 812. The next day, the cubes were embedded in the following resin mixture: Epon 812, 47%; dodecyl succinic anhydride, 27%; nadic methyl anhydride, 24%; and 2,4,6-tri (dimethylaminoethyl) phenol, 2%. The resin mixture was cured at 60°C for 2-3 days.

FIXATION OF CELLS FOR ELECTRON MICROSCOPY

For their use in immunoelectron microscopy, the cells were gently fixed and the secondary fixation in osmium tetroxide was omitted. The technique of fixation was as for vaccinia virus.

Two fixative agents were used in these studies: paraformaldehyde and glutaraldehyde. They were never utilized in combination. The concentration of the fixative agent and the time of fixation were: for paraformaldehyde, 2.5% for 30 min and 5% for 4 h; for glutaraldehyde, 1% and 2.5% for 30 min and 2 h.

Paraformaldehyde solution was prepared according to the method of Glauert (1965): 40 g of powdered paraformaldehyde was dissolved in 100 ml of distilled water in a water bath at 65°C. Clearing of the cloudy solution was done by adding a few drops of 10 N NaOH.

Commercial glutaraldehyde was purified by 4-5 washes in charcoal as recommended by Anderson (1967). To 200 ml of glutaraldehyde were added 35 g of activated charcoal (alkaline Norit A). The suspension was mixed and immediatly filtered on Buchner using Whatman #53 filter (hardened). The filtrate was again treated with charcoal in a proportion of 20% (w/w). This procedure was repeated until the ratio of absorbance at 235 nm/280 nm was less than 0.25.

The commercial glutaraldehyde had a ratio of absorbance of 17 and when 200 ml were purified by 4-5 washes in charcoal, the final product (about 50 ml) had a ratio of 0.20-0.22. Only freshly prepared glutaraldehyde was used for fixation.

The quantitation of glutaraldehyde was done using the following relationship: a 1M solution at 26°C has an absorbance reading at 280 nm of 6.0. This relation was used only when the material was relatively pure i.e. when the absorbance ratio (235 nm/280 nm) was less than 0.25.

GLYCOL METHACRYLATE EMBEDDING

Infected and non-infected cells were embedded in glycol methacrylate (GMA) as described by Leduc and Bernhard (1967).

The cells were fixed as described earlier and after embedding in Noble Agar, the material was transferred in a cold room where all the subsequent operations were performed. Before being embedded in GMA, the dehydrated agar cubes were soaked for 30 s in crystal violet (0.1% in 0.1M citric acid), to permit a better visualization of the cell pellet. The hardened blocks were kept at 4°C and brought up to room temperature before sectioning.

IMMUNOELECTRON MICROSCOPY

1) Purification of horse spleen ferritin

The following technique obtained from G. Nicholson (personal communication), was utilized to purify ferritin.

Horse spleen ferritin 6 times recrystallized (Cd free), was diluted to a concentration of 10 mg/ml with a solution of 2% $(NH_4)_2$ SO_4. To that, 34 ml of a solution of 20% $CdSO_4$ (anhydrous) was added for each 100 ml of diluted ferritin. This was left to crystallize overnight at 4°C. The crystals obtained were collected by centrifugation at 5000xg for 15 min and the pellet was dissolved to the original volume of the $(NH_4)_2$ SO_4 in a solution of 2% $(NH_4)_2$ SO_4 so that another crystallization could be performed.

After recrystallization, the crystals pelleted by centrifugation, were dissolved in a solution of 2% $(NH_4)_2SO_4$ identical to the original volume of ferritin at 10 mg/ml. To this was added an equal volume of a saturated solution of ammonium sulfate and the resulting solution was stored at 4°C for 1 h. The precipitate obtained was collected by centrifugation at 8000xg for 15 min and dissolved in the original volume of the saturated ammonium sulfate solution. The precipitation step for 1 h at 4°C was repeated a second time.

After collection, the precipitate was dissolved in a small volume of distilled water and dialysed overnight against cold running tap water. A final dialysis was done against 0.05M phosphate buffer pH 7.4 at 4°C. The dialysate was stored at -20°C.

The concentration of the ferritin solution was increased by centrifuging an aliquot of the dialysate at 75,000xg for 2 h. At the end of the centrifugation, the tubes contained 2 layers: the top 2/3 or 3/4 were colorless (apoferritin) and were removed with a syringe. The bottom pellet containing the concentrated ferritin, was allowed to dissolve in the remaining liquid. This step would approximately triple the concentration of the ferritin solution and was done prior to conjugation with gamma-globulin. The concentration of the final solution was determined by the Lowry method or by using the following relation: optical density at 440 nm x 0.65 x dilution factor = mg ferritin/ml (Nicholson, personal communication).

2) Preparation of the ferritin-gamma-globulin conjugate

The conjugation of ferritin to gamma-globulin was performed essentially as described by Siess et al (1971).

The ferritin and the globulin solutions were mixed to give the following final concentrations: globulin at 5 mg/ml and ferritin at 12 mg/ml, all in 0.1M phosphate buffer pH 7.3-7.4. The pH is very critical to achieve optimum yield of conjugate. To this solution was added purified glutaraldehyde to a final concentration of 0.035%. The conjugation was carried out for 1 h at room temperature.

This mixture was then brought to 25% saturation in ammonium sulfate. This solution was put at 4°C for 1 h. All the subsequent operations were done at 4°C. The precipitated conjugate was collected by centrifugation at 2000 rpm for 15 min and washed 3 times with a solution containing 25% $(NH_4)_2SO_4$ neutralized to pH 7.0 with ammonia (made just prior to use). The precipitate was dissolved in a small volume of 0.1M phosphate pH 7.2 and dialysed overnight against the same buffer at 4°C. The conjugate was stored at -20°C.

Before storage, the conjugate was analysed by immunodiffusion or by immunoelectrophoresis to check for the presence of active conjugate. The protein concentration of the conjugate solution was determined by the Lowry procedure.

It was found preferable to use fresh conjugate, since upon storage, the iron content of the ferritin would dissociate. Storage at 4°C is not recommended since the conjugated molecules will aggregate thus preventing free diffusion in the GMA.

3) Immunoferritin staining of infected cells

The staining of the infected cells, performed essentially as described by Kraehenbuhl and Jamieson (1972) is presented in Figure 2.

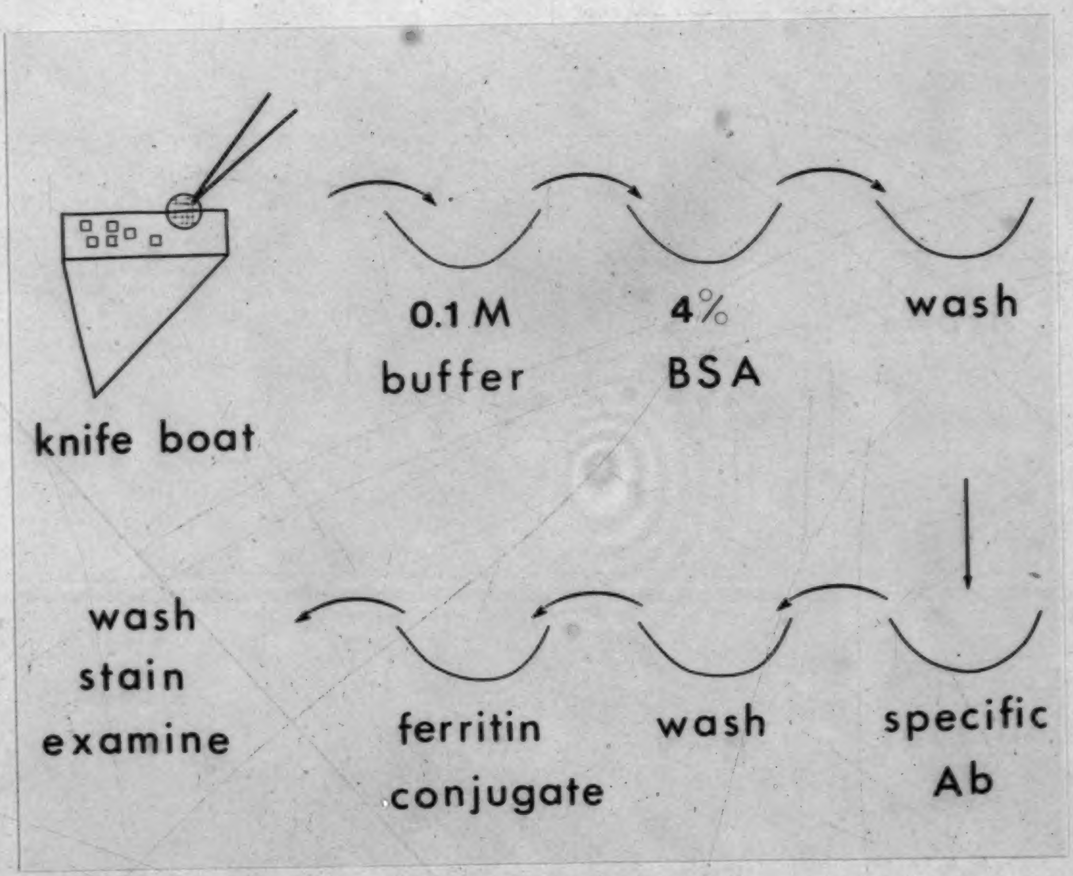
Sections of glycol methacrylate embedded material were allowed to swell in water for 10 min and then picked-up with a 200 mesh Formvar coated copper grid strenthened with carbon. The sections were then stained by inverting the grid onto the following solutions:

1) 0.1M phosphate buffer pH 7.2	5 min
2) 4% bovine serum albumin in buffer	10 min
3) specific antiserum diluted in PBS (1:5)	30 min
4) 3 washes in distilled water	10 min each
5) ferritin conjugate (0.5-1.5 mg/ml)	30 min
6) 3-5 washes in distilled water	10 min each
7) 2% uranyl acetate in water	20 min

After staining with uranyl acetate, the grids were dried and examined by electron microscopy. Photographs were taken and ferritin grains were counted directly from the negatives obtained.

FIGURE 2

Schematic representation of the various steps performed during staining of infected cells by the immunoferritin technique.


knife boat
0.1 M buffer
4% BSA
wash
specific Ab
wash
ferritin conjugate
wash
stain
examine

LIST OF SUPPLIERS

MEM (Gibco, Grands Island, N.Y.); Noble Agar (Difco Laboratories, Detroit, Michigan); cesium chloride (American Potash and Chemical Corporation, West Chicago, Illinois); sucrose (Fisher Scientific Company, Fair Lawn, N.J.); NP-40 (Shell Company, N.J.); sodium deoxycholate (Sigma Chemical Corporation, St Louis, Missouri); cethyl trimethyl ammonium bromide (Sigma Chemical Corporation, St Louis, Missouri); sodium dodecyl sulfate (Fisher Scientific Company, Fair Lawn, N.J.); agarose (Calbiochem, San Diego, Calf.); acrylamide (Matheson Coleman and Bell, Norwood, Ohio); bis-acrylamide (Eastman Kodak Co., Rochester, N.Y.); TEMED (Eastman Kodak Co., Rochester, N.Y.); dithiothreitol (Calbiochem, San Diego, Calif.); urea (Mann Research Chemical, New York, N.Y.); Coomassie Brilliant Blue R-250 (Schwarz/Mann, Orangeburg, N.Y.); periodic acid (Anachemia, Montreal); Sudan Black B (E.H. Sargent and Co., Chicago, Illinois); phosphotungstic acid (E. Fullam Inc., Schenectady, N.Y.); uranyl acetate (The British Drug Houses Limited, Poole, England); lead citrate (K&K Laboratories, Plainview, N.Y.); all the material used for embedding in Epon 812 were obtained from Fisher Scientific Company, Fair Lawn, N.J.; all the material used for embedding in glycol methacrylate were obtained from Polysciences, Inc., Warrington, Pa.; paraformaldehyde (Fisher Scientific Company, Fair Lawn, N.J.); glutaraldehyde (matheson Coleman and Bell, Norwood, Ohio); osmium tetroxide (Polysciences, Inc.,).

Warrington, Pa.); alkaline Norit-A (Fisher Scientific Company, Fair Lawn, N.J.); horse spleen ferritin (Polysciences, Ins., Warrington, Pa.).

RESULTS

CHARACTERIZATION OF THE "WYETH CALF ADAPTED" STRAIN OF VACCINIA VIRUS

In order to learn more about the growth characteristics of this particular strain of virus, it was essential to be able to quantitate the number of infectious units. The double overlay technique for plaque assay, described in Materials and Methods, gave countable plaques at 4 days of incubation. The size of the plaques ranged between 0.5-3.0 mm in diameter after a week of incubation.

GROWTH CURVE

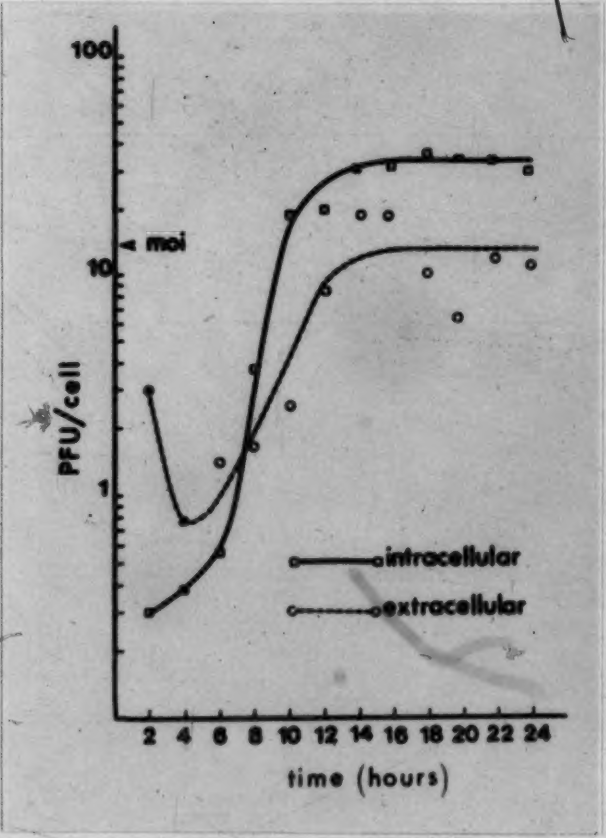
Since large quantities of viruses had to be grown, a growth curve was determined to give the time of harvest for maximum yield of vaccinia virus.

Cells were infected and the number of intracellular and extracellular infectious units (PFU) was determined every 2 h after infection, as described in Materials and ethods. The results are presented in Figure 3. After 2 h post-infection, about 25% of the inoculum is found in the medium (extracellular). Between 2-5 h after infection, the virus is in the eclipse period, indicated by the low number of PFU's both intracellularly and extracellularly. Then a logarithmic growth occurs between 6 to 12 h after infection. After this, the intracellular growth reaches

FIGURE 3

One-step growth curve of vaccinia virus in LLC-MK2.

Prescription bottles containing a monolayer of LLC-MK2 were infected at a moi of 13.5 PFU/cell. Every 2 h after infection, samples were taken and the amount of intracellular and extracellular PFU's per cell was determined by the plaque assay technique.


100
10
1
PFU/cell
moi
intracellular
extracellular
2 4 6 8 10 12 14 16 18 20 22 24
time (hours)

a plateau. The number of extracellular PFU's reaches also a plateau at about the same time. The input virus per cell was 13.5 PFU/cell, and the maximum production of viruses after infection was about 50 PFU/cell (total of extra and intracellular PFU's). Out of 50 PFU/cell, 12 were found extracellularly (24%).

RATIO OF VIRAL PARTICLES TO PLAQUE FORMING UNITS (VP/PFU)

Since this vaccinia virus strain was not known to have been passed in cell culture, it was necessary to find how well this strain could infect monkey kidney cells i.e. it was necessary to adapt this strain to the cell line, by repeated passages. To measure this "adaptation" the plaquing efficiency of the virus was determined by comparing the ratio of viral particles to plaque forming units during the passage of the virus in LLC-MK2. Intracellular viruses obtained by repeated freezing and thawing of cells infected during the passage of the virus in LLC-MK2, were used to determine the ratio of VP/PFU. The results presented in Table 1 show an increase in the number of PFU produced by the infected cells and a decrease in the ratio of viral particles to plaque forming units. This decrease in ratio indicates that the efficiency of infection increases with repeated passages in LLC-MK2. During this experiment the VP/PFU ratio decreased from 2000 to 130 between the 7th and 16th passage and reached a low of about 12 VP/PFU around the 17th and 18th passages. The growth curve experiment, the production of large quantities of viruses and the production of the stock virus were done using viruses which had been passed more than 20 times in cell culture.

PURIFICATION OF VACCINIA VIRUS

The technique of sucrose sedimentation velocity described in material and methods was used to purify large quantities of virus.

A typical centrifugation result, presented in Figure 4 shows the broad viral band when concentrated extracts were used (5 mg/ml). Analysis of the collected material was done by electron microscopy, absorbance (ratio of 260 nm/ 280 nm) and immunodiffusion to determine the purity of the viral preparation. Electron microscopy (negative contrast staining) showed typical brick-shaped viruses (Fig. 5). Very little cellular debris could be detected. The ratio of absorbance at 260 nm/ 280 nm was 1.22 and remained constant when three successive centrifugations in sucrose were performed. This constant ratio indicates that the purity of the preparation did not increase by repeated centrifugations. Immunodiffusion analysis failed to detect host cell material or soluble viral antigens present in the dialysed suspension of whole virions.

The results of an experiment involving two successive centrifugations in a sucrose gradient are presented in Table 2. The number of PFU after the second sucrose

TABLE 1

Plaquing efficiency of vaccinia virus in LLC-MK2

Passage#*	VP/ml	PFU/ml	Ratio(VP/PFU)
7	1.2×10^8	6×10^4	2000
15	4.1×10^8	1×10^6	400
16	5×10^{10}	3.1×10^8	130

* The passage of vaccinia virus in LLC-MK2 was done the following way: 3 ounce bottles containing a confluent layer of cells were infected with 0.5 ml of the original viral suspension. When the cell sheet showed complete cytopathic effect, the bottles were frozen and thawed 5 times and 0.5 ml of this suspension was used to inoculate other 3 ounce bottles.

FIGURE 4

Purification of vaccinia virus by sedimentation velocity in a sucrose gradient.

A crude suspension of vaccinia virus, prepared as described in material and methods was put on a 20-40% linear sucrose gradient, and centrifuged at 25,000xg for 45 min. The viral band shown here, was collected from the top with a syringe.

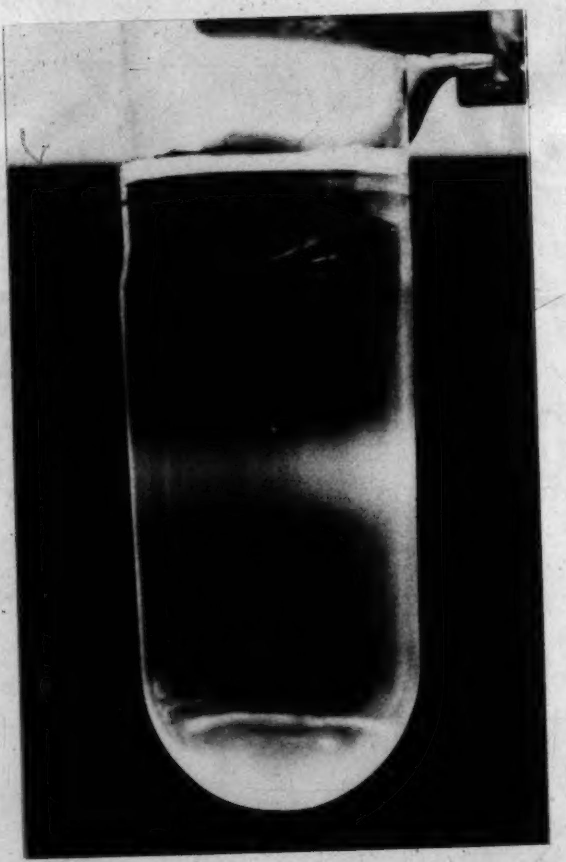


FIGURE 5

NEGATIVELY STAINED PREPARATION OF VACCINIA VIRUS

Vaccinia virus purified by sucrose sedimentation velocity was dialysed overnight against PBS at 4°C. A sample of the dialysed suspension was examined by negative contrast staining. x 60,000

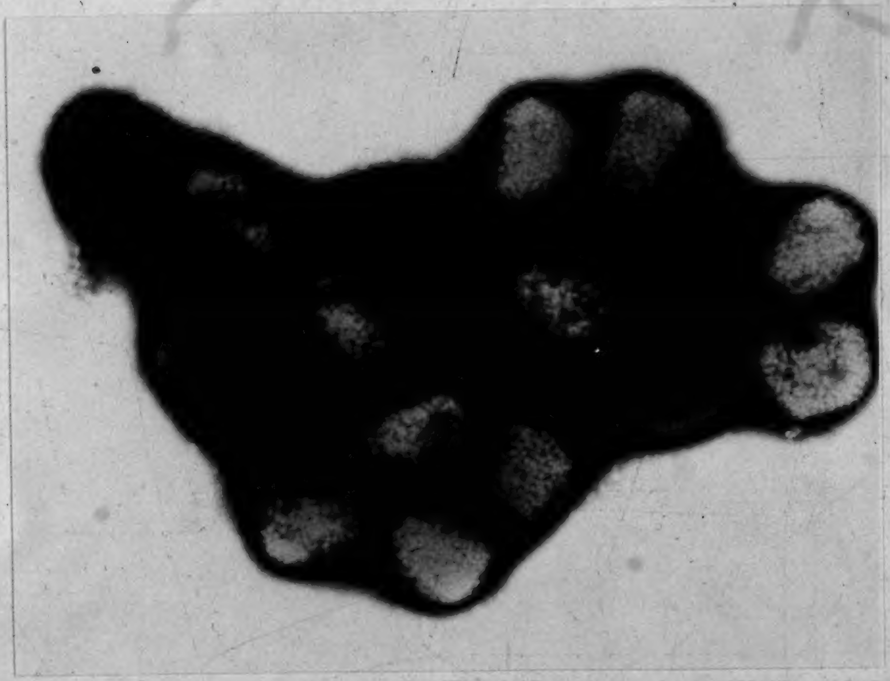


TABLE 2

Purification of vaccinia virus by sedimentation velocity in a sucrose gradient (1)

Sample	PFU(total)	VP(total)	VP/PFU	Ratio(260/280)	mg protein	Yield(2)
Crude(3)	1.3×10^{11}	1.5×10^{12}	12	------	------	100%
Before 1st purification (4)	------	8.1×10^{11}	--	------	------	63%
After 1st purification	1×10^{10}	3.8×10^{11}	38	1.22	12	26%
After 2nd purification	4.2×10^{8}	1.8×10^{11}	430	1.20	5.9	14%

(1) A total of 20 roller bottles were used

(2) The number of viral particles in the crude sample is taken as 100%

(3) Sample after homogenization

(4) Sample after sonication and centrifugation at 3000xg for 20 min.

purification represents less than 0.3% of the PFU's present in the crude preparation. On the other hand, the number of viral particles represent 12% of that present in the crude preparation. The ratio of VP/PFU, because of this large decrease in PFU, increased from 12 to 430 during purification. The ratio of absorbance (260/280 nm) did not vary significantly between the two centrifugations. The amount of recovered virus decreased by more than 50% between the first and second sedimentation velocity in sucrose.

After the first centrifugation in sucrose, a total of 12 mg of virus was obtained. Since this represents 26% of the original yield of viral particles, it can be estimated that one roller bottle could yield 2.3 mg of vaccinia virus in the crude sample.

Because of the loss in PFU's without a significant increase in purity, it was decided to omit the second sucrose centrifugation step.

All the viral preparations used in these studies were purified by one centrifugation in a sucrose gradient.

POLYPEPTIDE COMPOSITION OF VACCINIA VIRUS

Samples of vaccinia virus were solubilized in SDS and subjected to electrophoresis in SDS-acrylamide gel as described in Materials and Methods.

Figure 6 shows a densitometer tracing of a Coomassie Blue stained gel of whole vaccinia virus, purified by sucrose sedimentation velocity. The maximum number of resolvable polypeptide bands* obtained, was 33. The number of bands and the pattern of the densitometer tracing were quite reproducible. In a few instances the number of bands and the relative intensity of the stained band would be different. The reasons were that some of the bands with very close relative mobility were not adequately resolved on the gel and also the background in some parts of the gel was higher than usual.

As presented in Figure 6, the densitometer tracing indicates that VP-2, VP-4, VP-6, VP-10 and VP-17 are the most abundant polypeptides+. VP-2, VP-4 and VP-6 are actually groups, composed of 2-3 polypeptide bands having a close relative mobility. VP-2 is composed of 3 polypeptides;

---

* The bands seen on the gels will be referred to as polypeptide bands. The use of the word polypeptide band is for comparison with other work and the limits and the implications of this terminology will be discussed later.

+ The numbers of the polypeptide bands were originally those described by Joklik and Holowczak (1967a) and will be used in this work for the sake of comparison. VP stands for viral protein.

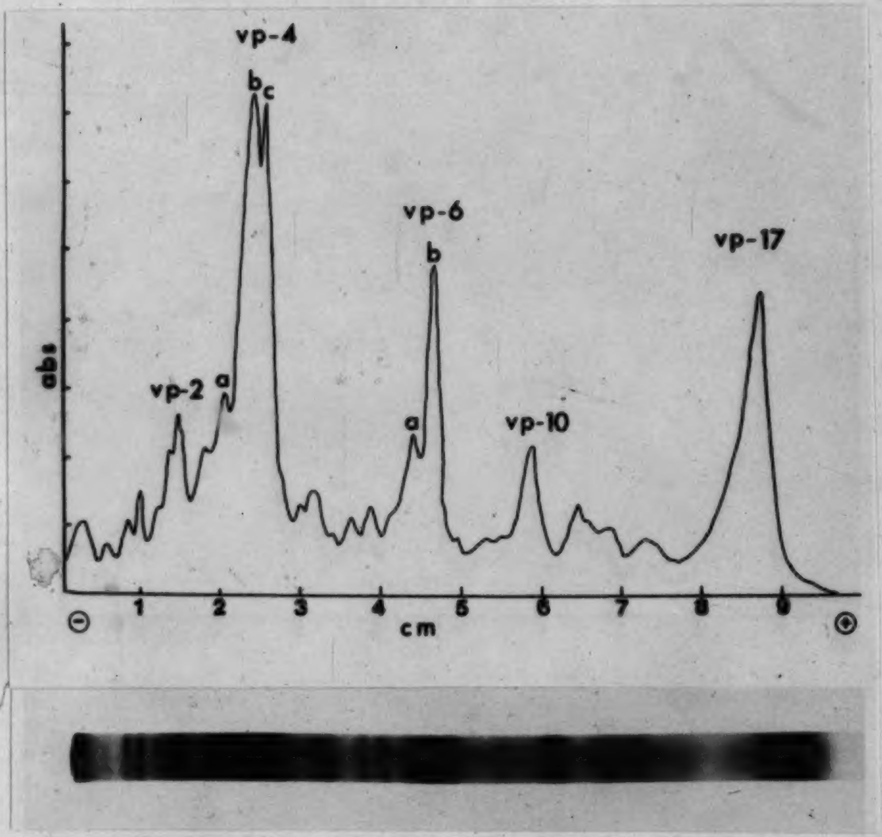
VP-4 is composed of 3 polypeptides called VP-4a, VP-4b and VP-4c; finally VP-6 is made of 2 polypeptides: VP-6a and VP-6b. VP-10 was always seen as a single stained band. VP-17 appears as a single polypeptide band on the densitometer tracing, but some of the gels indicate that this region of the gel may contain more than one polypeptide component (Fig. 6, 10 and 11). Since the other polypeptide bands composing the virus are not as abundant as those mentioned, this study will be concerned mainly with the more abundant polypeptide bands, i.e. those which have been numbered previously.

Since the number of polypeptide bands found was more numerous than that originally described by Joklik and Holowczak (1967a), it was thought that sedimentation in a sucrose gradient did not purify the viral population adequately, and that some of the polypeptide bands might originate from host material or could be viral soluble components. To test the possibility of having a contaminated viral preparation, a purified preparation was purified again by sucrose sedimentation velocity and a third time by cesium chloride density gradient centrifugation. After each purification, a sample was taken, analysed for purity as described in materials and methods and solubilized in SDS, dithiothreitol and urea. The number of polypeptide bands was determined by SDS gel electrophoresis. The three purified viral preparations contained the same number of polypeptide bands and the densitometer tracings

FIGURE 6

Densitometer tracing of a gel containing solubilized purified vaccinia virus.

Purified vaccinia virus was solubilized and electrophoresed as described in material and methods. The gel was stained in Coomassie Blue and scanned at 585 nm. The direction of migration is from left to right (anode). This will apply for all the densitometer tracings.


vp-4
b
c
vp-6
b
a
vp-2
a
vp-10
vp-17
abs
1
2
3
4
5
6
7
8
9
cm
⊖
⊕

of the gels were identical to each other. This would indicate that repeated centrifugations in density gradients did not give different results in the polypeptide composition of vaccinia virus.

Since this last experiment did not exclude the possibility of having soluble contaminants with sedimentation characteristics similar to vaccinia virus, the samples were also analysed by discontinuous disc gel electrophoresis. Vaccinia virus because of its size, does not penetrate a 7.5% acrylamide gel, so any material migrating in the gel would be considered as a contaminant. When 200 µg of purified vaccinia virus were examined by disc gel electrophoresis, none of the samples showed the presence of any stainable material in the gel, indicating that no soluble contaminants were present in the purified preparations, whether they had been purified once or twice by sucrose sedimentation velocity.

To exclude the possibility that some of the high molecular weight polypeptides were not reaggreagated sub-units normally held by disulfide bonds, the purified virus was solubilized in the presence of various reducing agents. Mercapto-ethanol (1%), dithiodiethanol (0.1M), were compared to dithiothreitol (5 mM) and the resulting densitometer tracings of the gels were found to be identical to each other.

CHARACTERIZATION OF THE POLYPEPTIDES OF VACCINIA VIRUS

1) Molecular weight determination of the major polypeptides

The molecular weights of the most abundant polypeptides of vaccinia virus, determined as described in Materials and Methods, are presented in Table 3. There is a wide range of polypeptides sizes. The highest molecular weight observed, was estimated to be around 90,000 daltons (VP-2) while the lowest was VP-17 with a molecular weight of 11,000 daltons. The must abundant group of polypeptides, the VP-4's, had a molecular weight of about 70,000 daltons.

2) Glycopeptide and lipopeptide characterization

To further characterize the viral polypeptides, specific staining techniques described in materials and methods were employed.

None of the polypeptide bands stained positively for lipids. Eight polypeptide bands gave indications that they were glycopeptides. Immediately after the staining reaction only the VP-17 band stained intensely. Four days later, more colored bands would appear on the gel and reached maximum intensity in 6 days.

A densitometer tracing of a gel stained for glycopeptides is presented in Figure 7. The bands that stained most intensely are the VP-6's and VP-17. Other stained glycopeptide bands migrated in the VP-4 area and on either side of the VP-6's. The high molecular weight glycopeptide band (migrating close to the top of the gel) appeared in only two out of six gels stained for carbohydrates.

TABLE 3

Molecular weights of the more abundant polypeptides of vaccinia virus determined by SDS gel electrophoresis.

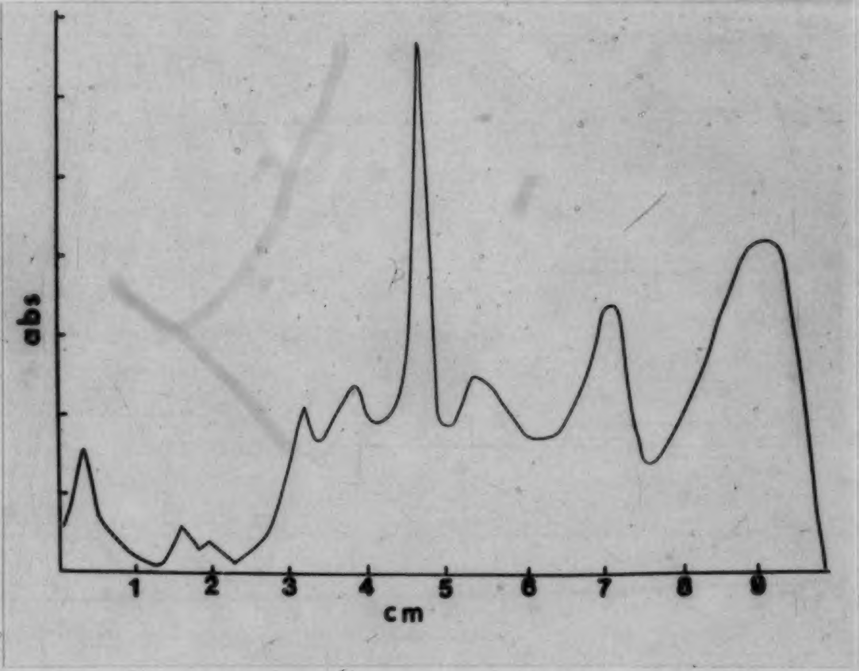
Polypeptide band	Molecular weight* (daltons)
VP-2 (major peak)	90,000
VP-4a	75,000
VP-4b	68,000
VP-4c	65,000
VP-6a	36,000
VP-6b	32,000
VP-10	22,500
VP-17	11,000

* Average of 4-6 different determinations.

FIGURE 7

Densitometer tracing of a gel containing solubilized vaccinia virus and stained for the presence of glycopeptides.

Purified vaccinia virus was solubilized and electrophoresed as described in materials and methods. The gel was stained by the periodic acid Schiff's reagent technique to detect glycopeptides. After 6 days, the gel was scanned at 545 nm.


abs
1
2
3
4
5
6
7
8
9
cm

POLYACRYLAMIDE GEL ELECTROPHORESIS ANALYSIS OF PROTEINS EXTRACTED FROM VACCINIA VIRUS

1) Chemical extraction of vaccinia virus

Purified virus (without disulfide bond reduction) was subjected to extraction by 8M urea or 6N guanidine hydrochloride as described in material and methods. An alkaline treatment at pH 10.5 for 3 h was also performed on vaccinia virus.

The supernatants containing material extracted from the virus were analysed by SDS gel electrophoresis. Since constant amount of virus were utilized for the extraction, and since identical volumes of samples were electrophorezed, one could evaluate semi-quantitative differences in the amount of material released from the virus by the various chemical treatments.

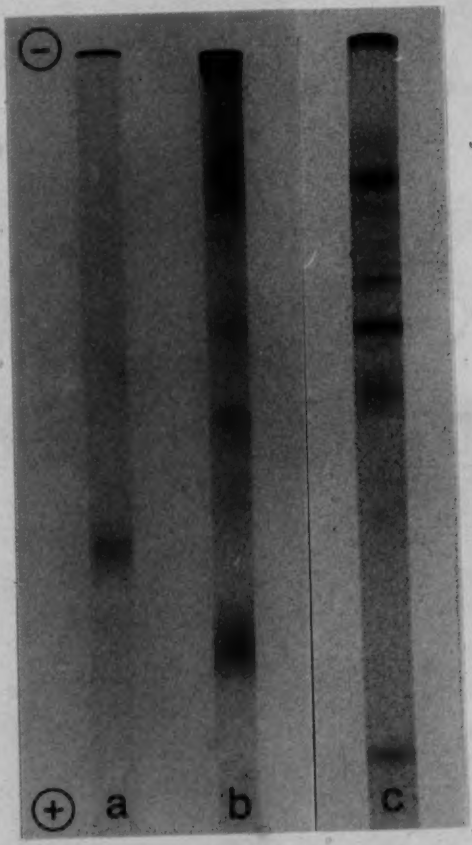
The results shown in Figure 8 indicate that alkaline treatment (c) released the most proteins while guanidine hydrochloride (a) released the least. Treatment of the virus at pH 10.5 released 12 to 14 polypeptides of differing molecular weights. The most abundant polypeptide released, migrated between the VP-4 and the VP-6 group. Some of the VP-4, VP-6 and VP-17 polypeptides were also released in small amounts.

Treatment of the virus with guanidine hydrochloride failed to released any significant amount of material except for a polypeptide migrating near VP-10. On the

FIGURE 8

Photograph of gels containing chemical extracts of vaccinia virus analysed by SDS gel electrophoresis.

SDS gel electrophoresis of chemical extracts of vaccinia virus. a) extracted by 6N guanidine hydrochloride b) extracted by 8M urea c) extracted by alkaline treatment. Equal amounts of virus were extracted in all cases. Direction of migration of those gels only, is from top to bottom.


−
+
a
b
c

other hand, treatment with 8M urea released 8 polypeptides. All but a polypeptide migrating near the VP-17 polypeptide were in small amounts. This includes the polypeptides migrating around the VP-4 and the VP-6 group of polypeptides.

Controls in which purified viruses were incubated in phosphate buffer 0.01M pH 7.2, for 3 h at 37°C did not show any stainable material in SDS gels.

2) Detergent extraction of vaccinia virus.

Since it was hoped to find a means of disrupting vaccinia virus without completely destroying the native structure of the sub-units, detergents represented a possible way of selectively removing sub-units from the viral structure. It was decided to use representatives of non-ionic, cationic and anionic detergents. Non-ionic detergents selected were NP-40, Triton X-100, Brij 35, Brij 58, Tween 60 and Tween 80. An anionic detergent was SDC while a cationic detergent was CTAB. As mentioned in Materials and Methods, the detergents were used at the same concentration in the presence of identical amounts of virus. One could therefore compare the amount of protein released, when aliquots of the same volume were analysed by SDS gel electrophoresis.

Only NP-40 and Triton X-100, among the non-ionic detergents, extracted significant amounts of material from the virions. Both of them removed identical types of polypeptides as shown in Figure 9. The material solubilized by the non-ionic detergents is composed mainly of the VP-6a and VP-6b. Other less abundant constituents released were situated around the VP-4 area, between VP-4 and VP-6 and finally some polypeptide bands migrated as VP-10 and VP-17

The polypeptides released by SDC are shown in Figure 10. At least 25 polypeptide bands were visually counted on the gel. All the polypeptides migrating between VP-4 and VP-17 are well solubilized by SDC. The higher molecular weight polypeptides (migrating between the origin and VP-4) were not as well released by the detergent. The most abundant polypeptides released were the VP-6a and VP-6b.

The type of polypeptides extracted by the cationic detergent CTAB is shown in Figure 11. The main difference between this pattern and the one presented in Figure 6 (complete solubilization of the virus) is in the amount of some extracted polypeptides. The total number of polypeptides visually counted was 33. Among the polypeptides not well extracted were VP-17 (which appear as 2 stained bands), and most of the higher molecular weight polypeptides.

3) Extraction of vaccinia virus by detergents and a disulfide bond reducing agent.

The effect of a disulfide bond reducing agent in the effectiveness of detergents, was also examined. In all cases, 5 mM dithiothreitol was used as the reducing agent. The results are presented in Figures 12, 13 and 14.

There were no obvious differences between the types of polypeptides released in the presence of dithiothreitol, except for a lower level of background in the upper parts of the gels. As shown in these figures, the low background gave rise to a much better resolution of the polypeptide bands.

FIGURE 9

SDS gel electrophoresis analysis of extracts of vaccinia virus by non-ionic detergents.

Purified vaccinia virus was extracted by NP-40 and Triton X-100 as described in Materials and Methods and subjected to electrophoresis in SDS gel. The densitometer tracing shows the results obtained with the NP-40 extract. The bottom photograph of an SDS gel shows the polypeptide pattern obtained from a Triton X-100 extract.

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2) Detergent extraction of vaccinia virus.

Since it was hoped to find a means of disrupting vaccinia virus without completely destroying the native structure of the sub-units, detergents represented a possible way of selectively removing sub-units from the viral structure. It was decided to use representatives of non-ionic, cationic and anionic detergents. Non-ionic detergents selected were NP-40, Triton X-100, Brij 35, Brij 58, Tween 60 and Tween 80. An anionic detergent was SDC while a cationic detergent was CTAB. As mentioned in Materials and Methods, the detergents were used at the same concentration in the presence of identical amounts of virus. One could therefore compare the amount of protein released, when aliquots of the same volume were analysed by SDS gel electrophoresis.

Only NP-40 and Triton X-100, among the non-ionic detergents, extracted significant amounts of material from the virions. Both of them removed identical types of polypeptides as shown in Figure 9. The material solubilized by the non-ionic detergents is composed mainly of the VP-6a and VP-6b. Other less abundant constituents released were situated around the VP-4 area, between VP-4 and VP-6 and finally some polypeptide bands migrated as VP-10 and VP-17

The polypeptides released by SDC are shown in Figure 10. At least 25 polypeptide bands were visually counted on the gel. All the polypeptides migrating between VP-4 and VP-17 are well solubilized by SDC. The higher molecular weight polypeptides (migrating between the origin and VP-4) were not as well released by the detergent. The most abundant polypeptides released were the VP-6a and VP-6b.

The type of polypeptides extracted by the cationic detergent CTAB is shown in Figure 11. The main difference between this pattern and the one presented in Figure 6 (complete solubilization of the virus) is in the amount of some extracted polypeptides. The total number of polypeptides visually counted was 33. Among the polypeptides not well extracted were VP-17 (which appear as 2 stained bands), and most of the higher molecular weight polypeptides.

3) Extraction of vaccinia virus by detergents and a disulfide bond reducing agent.

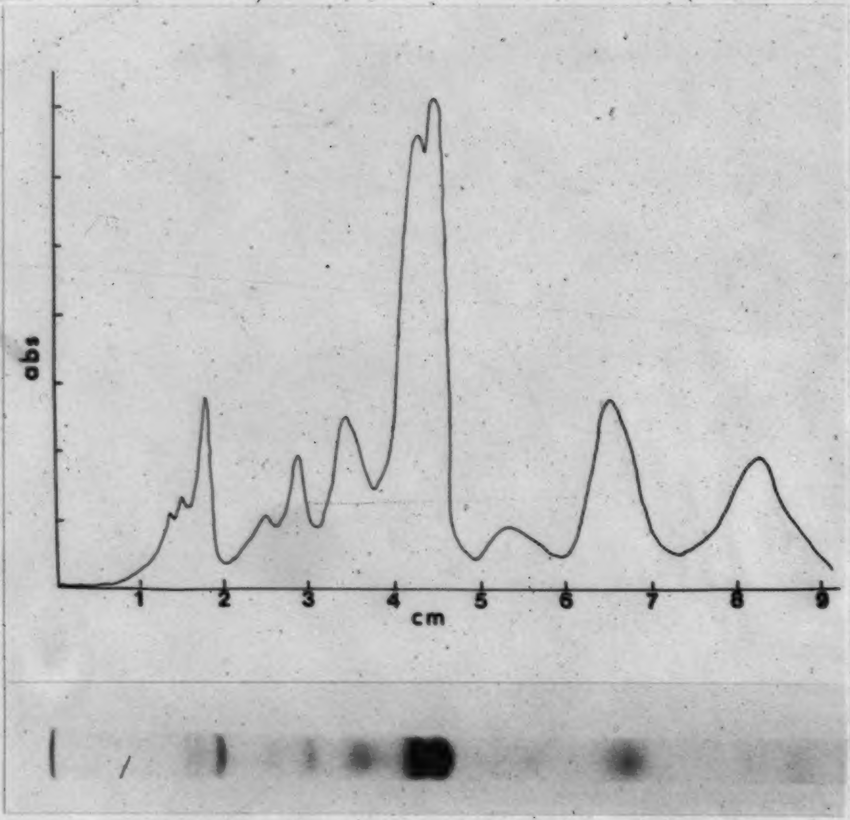
The effect of a disulfide bond reducing agent in the effectiveness of detergents, was also examined. In all cases, 5 mM dithiothreitol was used as the reducing agent. The results are presented in Figures 12, 13 and 14.

There were no obvious differences between the types of polypeptides released in the presence of dithiothreitol, except for a lower level of background in the upper parts of the gels. As shown in these figures, the low background gave rise to a much better resolution of the polypeptide bands.

FIGURE 9

SDS gel electrophoresis analysis of extracts of vaccinia virus by non-ionic detergents.

Purified vaccinia virus was extracted by NP-40 and Triton X-100 as described in Materials and Methods and subjected to electrophoresis in SDS gel. The densitometer tracing shows the results obtained with the NP-40 extract. The bottom photograph of an SDS gel shows the polypeptide pattern obtained from a Triton X-100 extract.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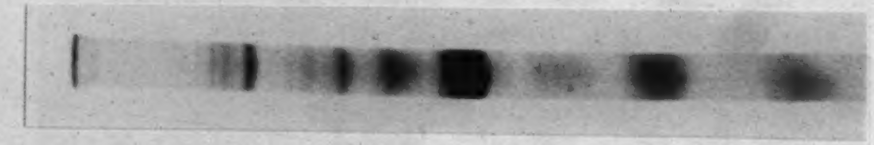

FIGURE 10

Densitometer tracing of a SDS gel containing solubilized polypeptides, extracted by SDC.

Purified vaccinia virus was extracted by SDC as described in Materials and Methods. The extracted proteins were solubilized in SDS and subjected to electrophoresis in SDS.

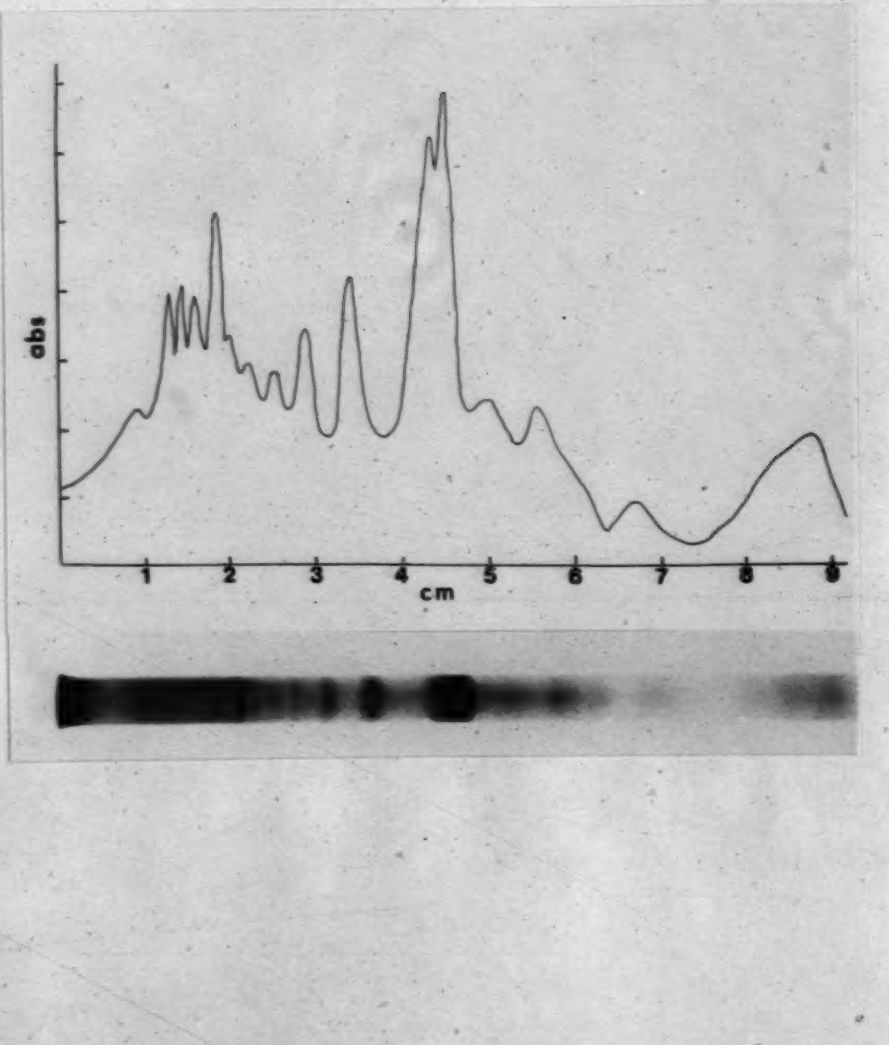
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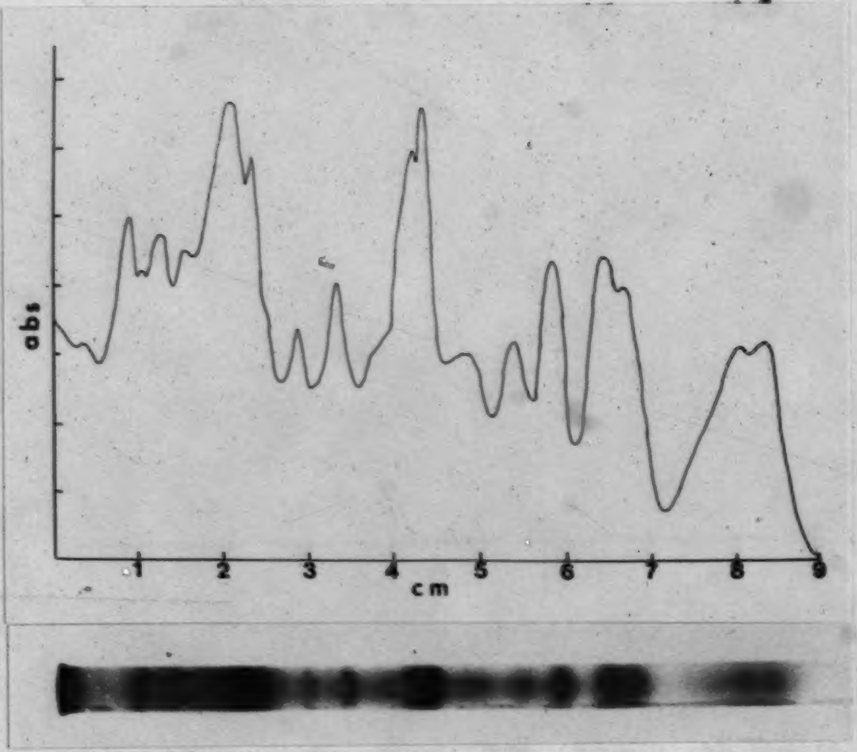
FIGURE 11

Densitometer tracing of an SDS gel containing solubilized polypeptides, extracted by CTAB.

Purified vaccinia virus was extracted by CTAB as described in Materials and Methods. The extracted proteins were solubilized in SDS and subjected to electrophoresis in SDS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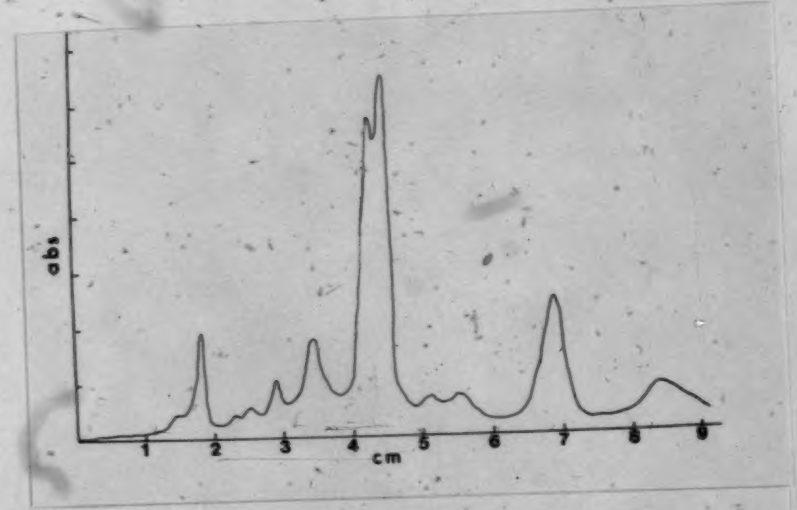
Densitometer tracings of SDS gels containing viral extracts obtained with the use of detergents and 5 mM dithiothreitol, and analysed by SDS gel electrophoresis.

FIGURE 12 (top) : 0.5% NP-40 extract

FIGURE 13 (middle): 0.5% SDC extract

FIGURE 14 (bottom): 0.5% CTAB extract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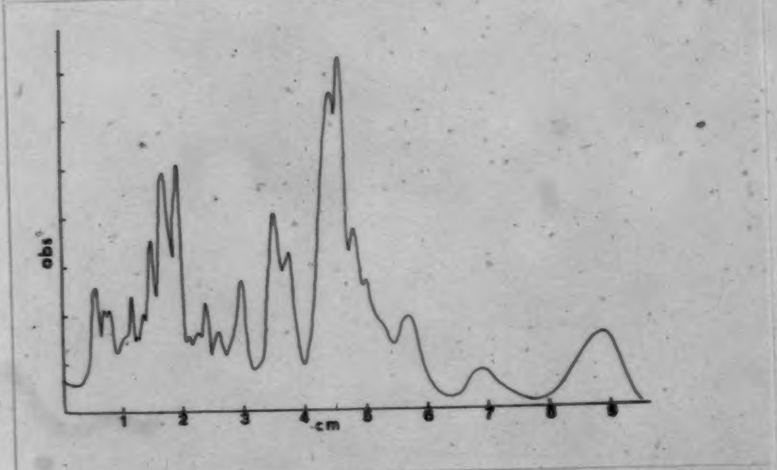
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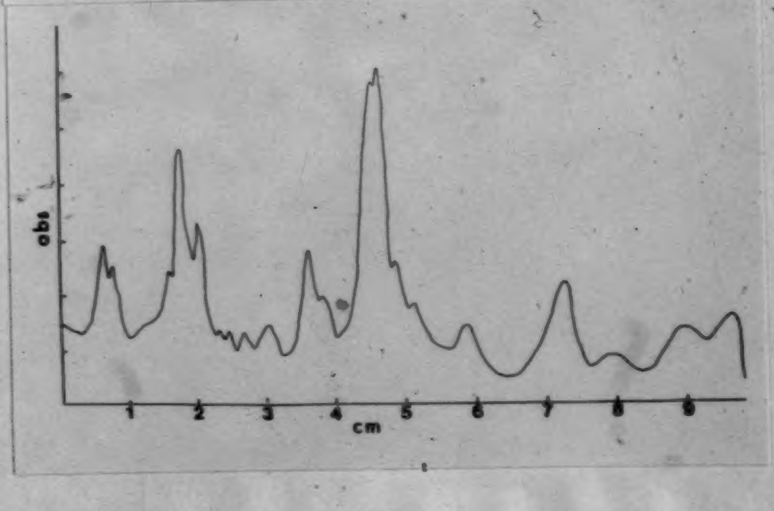
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AMOUNT OF PROTEIN EXTRACTED BY DETERGENTS FROM VACCINIA VIRUS.

After extraction residual virus was removed by high speed centrigugation (40,000xg, 90 min), and the supernatant analysed for protein content by the Lowry procedure. The results obtained are presented in Table 4.

NP-40, SDC and CTAB are increasingly more effective in solubilizing protein from vaccinia virus. The effect of the detergents on the solubilizable protein of the virions was found to be inversely proportional to the molecular weight of the detergents. The lower the molecular weight of a detergent the more protein was solubilized. (Table 4).

As indicated in Table 4, the use of a disulfide bond reducing agent increased the amount of protein solubilized by a detergent. In the case of NP-40 and SDC, there is a net increase of 15% while for CTAB, the increase is only 3%. SDS with or without a reducing agent completely solubilized the virions. Higher concentration of detergents or longer incubation period did not increase significantly the amount of protein solubilized.

GEL DIFFUSION ANALYSIS OF THE PROTEINS EXTRACTED BY DETERGENTS FROM VACCINIA VIRUS

The supernatants containing the solubilized viral proteins were analysed by microimmunodiffusion against whole vaccinia virus antiserum. The result of an analysis by immunodiffusion, of an NP-40 extract, is presented in Figure 15.

TABLE 4

AMOUNT OF PROTEIN SOLUBILIZED BY DETERGENTS FROM VACCINIA VIRUS

Detergent	% protein solubilized without DTT*	% protein solubilized with DTT	Molecular weight of detergent
NP-40	26	41	602
SDC	51	66	414.6
CTAB	76	79	364.5
SDS	100	100	288.4

* DTT: dithiothreitol, used at a concentration of 100 mM, only for this particular experiment.

After 48 h of diffusion, one major band of precipitation was seen. Later (72 h), two other faint precipitin bands became visible. These two faint bands as indicated in the figure, seem to be non-specific precipitin bands, due to reaction between the extract and substances present even in the antiserum to non-viral antiques (arrows).

When extracts of ionic detergents (SDC and CTAB) were examined by microimmunodiffusion, a complex pattern of non-specific precipitation was obtained. Since precipitation would occur between the detergent alone when tested against both viral and non-viral antigens it was concluded that the detergents were responsible for the non-specific precipitation. Thus, interpretation of the precipitin pattern obtained with ionic detergent extracts was prevented. Nevertheless, the immunodiffusion results indicated that NP-40 could extract a component from the virions without denaturing its antigenicity.

ANALYSIS OF VACCINIA VIRUS AFTER EXTRACTION BY DETERGENTS

1) Analysis of the extracted viral particles by SDS gel electrophoresis.

After extraction, the residual viruses pelleted by high speed centrifugation as described in Materials and Methods, were analysed by SDS gel electrophoresis to determine whether or not some of the components were totally and selectively extracted by the detergents. Also, examination of the extracted viral particles might give us

FIGURE 15

Microimmunodiffusion analysis of an NP-40 extract from vaccinia virus.

Microimmunodiffusion analysis of a dialysed 0.5% NP-40 extract, from purified vaccinia virus.

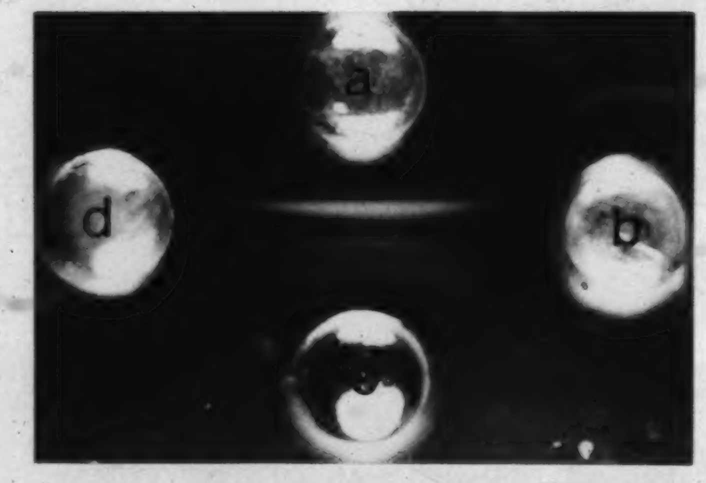
a) dialysed NP-40 extract

b) pre-immune serum

c) whole vaccinia virus antiserum

d) pre-immune serum

All the sera were used undiluted. The photograph was taken after 5 days of diffusion at room temperature.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some indication of the mode of action of these detergents on vaccinia virus.

Gel electrophoresis analysis of NP-40, SDC and CTAB extracted vaccinia virus are presented in Figures 16, 17 and 18. From the densitometer tracings, one can conclude that no polypeptides were selectively extracted from the virus; only Figure 18 shows some indication that the VP-6 group might be completely extracted from the virus by CTAB. The most abundant polypeptide group, VP-4, is left in large amounts in all cases. Also left in large amounts, but not, regarded as a major constituant of vaccinia virus, is the VP-17 polypeptide. The group of VP-6 polypeptides is not well released by SDC.

When the gels were examined visually there was little difference between the number of polypeptides not solubilized by NP-40 (Fig. 16) and SDC (Fig. 17). The densitometer tracings also resembled the one presented in Figure 6.

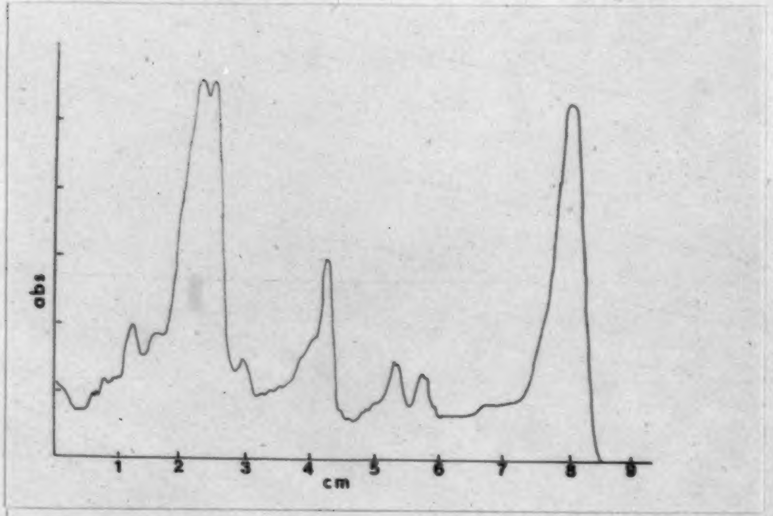
Figure 18 shows the pattern of polypeptides not solubilized by CTAB. As expected from the quantity and the type of polypeptides removed from the virus, the major polypeptides left are the VP-4 group plus some higher molecular weight polypeptides and VP-17. Most of the polypeptides migrating between the VP-4 group and VP-17 have been well solubilized by CTAB except for small amounts of VP-6a and VP-10.

Densitometer tracings of SDS gels containing solubilized viral particles, after extraction by detergents.

FIGURE 16 (top): after NP-40 extraction

FIGURE 17 (middle): after SDC extraction

FIGURE 18 (bottom): after CTAB extraction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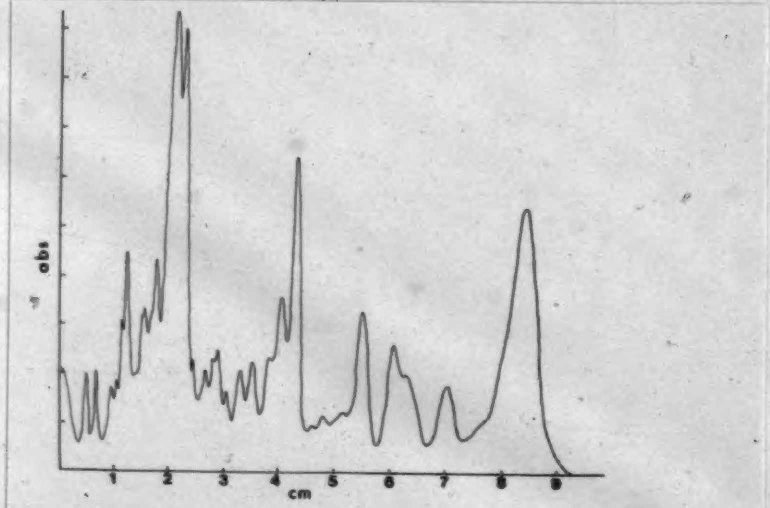
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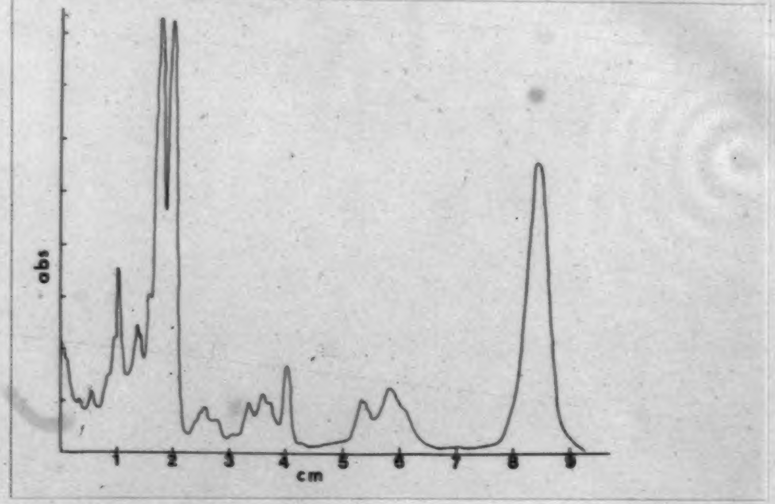
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2) Analysis of the extracted viral particles by electron microscopy

To correlate the alterations caused to the virus with the type of polypeptides extracted by the various detergents, an electron microscopic examination of the extracted viral particles was performed.

To study surface changes, the technique of negative contrast staining was used. Internal and surface alterations were investigated by the technique of ultramicrotomy.

a) Negative contrast staining

Immediately after extraction by the detergents, the virus was examined by negative staining as described in materials and methods. Vaccinia virus incubated in buffer was used as a control. The results are shown in Figure 19. A low magnification view of the virus indicates no apparent changes caused by 3 h of incubation at 37°C. The virus still retains its normal brick-shaped appearence and one can see the tubules covering the surface.

The alterations caused to vaccinia virus by NP-40 are shown in Figures 20, 21 and 22. None of the viral particles had retained their typical tubule covered surface. All the particles were permeable to the stain as presented in Figure 20. These particles resemble somewhat the "C" type particles described by Westwood et al. (1964).

In some cases, the virus were seen lying in a different plane of view. The arrow in Figure 20 points to such a particle. It shows the typical dumbell core flanked by the two lateral bodies. The fact that NP-40 permits a better penetration of the stain inside the virus helps in visualizing the "S" shape organization of the nucleoprotein complex residing in the viral core (arrow in Figure 21).

Another type of alteration seen, is shown in Figure 22. The smooth appearance of the viral surface as seen in the controls has changed. Instead, a jagged or toothed edge appearance is seen indicating surface alteration. This kind of alteration was seen in less than 10% of the examined particles because the orientation of the virus has to be the right way to show this toothed edge.

When vaccinia virus was examined after extraction by SDC, two disctinct types of alterations were observed. The most common effect seen (60% of the viral particles examined) is presented in Figure 23 in which the surface topography has been completely changed. The edge of the viral particles do not suggest the presence of tubules as seen in the controls, but rather they indicate a much smoother surface. The penetration of the particles by the stain indicates that the core or at least its content have been altered by the detergent. Both particles in Figure 23 show a core with a shrunken or coagulated appearance.

The other type of alteration observed in 30% of the viral particles examined is shown in Figure 24. The negative stain did not penetrate the viral particles. This figure shows a viral particle which might be either a core or a viral particle devoid of its surface protein coat. The latter hypothesis is most likely since cores have jagged edges (Pogo and Dales, 1969) while this particle present a smooth surface.

The material left after CTAB extraction was also examined by negative staining. Figure 25 shows irregular masses of material that seem to have no structural arrangements, alghough brick-shaped or rectangular forms can be seen at the edges of these aggregates. Even if there are little structural features left, one can observe striations or lines associated with these aggregates of extracted viral particles. Figure 26 shows a high magnification of those aggregates. The lines seen in Figure 25 seems to have a parallel orientation. However, no distinctive viral particles or viral structural components could be detected.

This study was also performed using virus treated with detergents and dithiothreitol (DTT). The viral morphology as observed in the electron microscope was not changed over and above that caused by the detergent alone. It is concluded that DTT did not significantly enhance the action of the detergents.

FIGURE 19

Electron micrograph of control (untreated) vaccinia virus negatively stained with PTA.

Purified vaccinia virus preparation was incubated for 3 h at 37°C, in 0.01 phosphate buffer pH 7.4. x 53,500

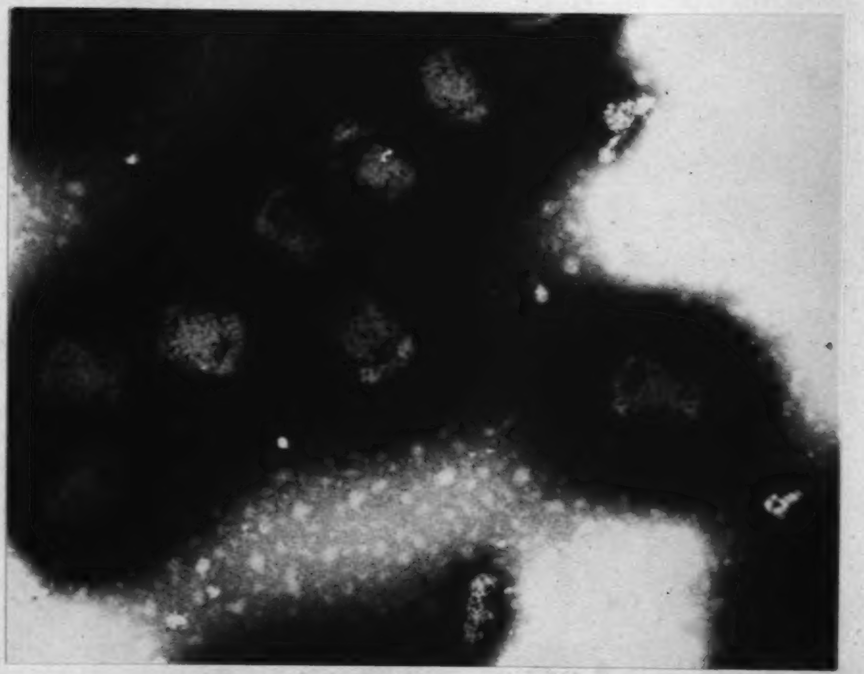


FIGURE 20

Electron micrograph of vaccinia virus, negatively stained after NP-40 treatment.

Vaccinia virus was treated with 0.5% NP-40 for 3 h at 37°C. x 111,000

FIGURE 21

Electron micrograph of vaccinia virus negatively stained after NP-40 treatment.

Vaccinia virus was treated with 0.5% NP-40 for 3 h at 37°C. This photograph shows the "S" shape of the nucleoprotein complex. x 144,000

FIGURE 22

Electron micrograph of vaccinia virus, negatively stained after treatment with NP-40

Viral particles after extraction by 0.5% NP-40. This photograph shows the "toothed edge" of the particles. x 111,000

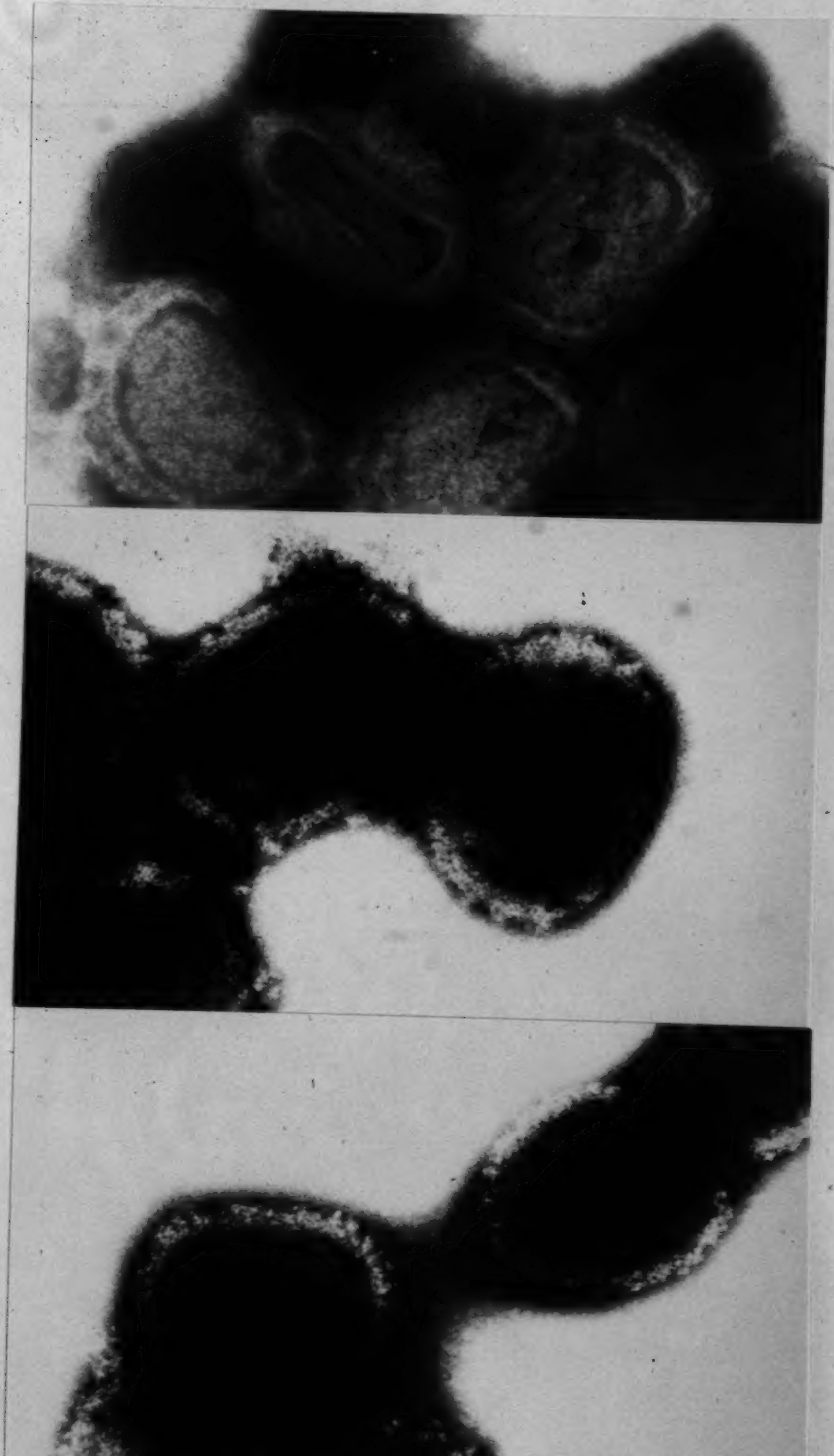


FIGURE 23

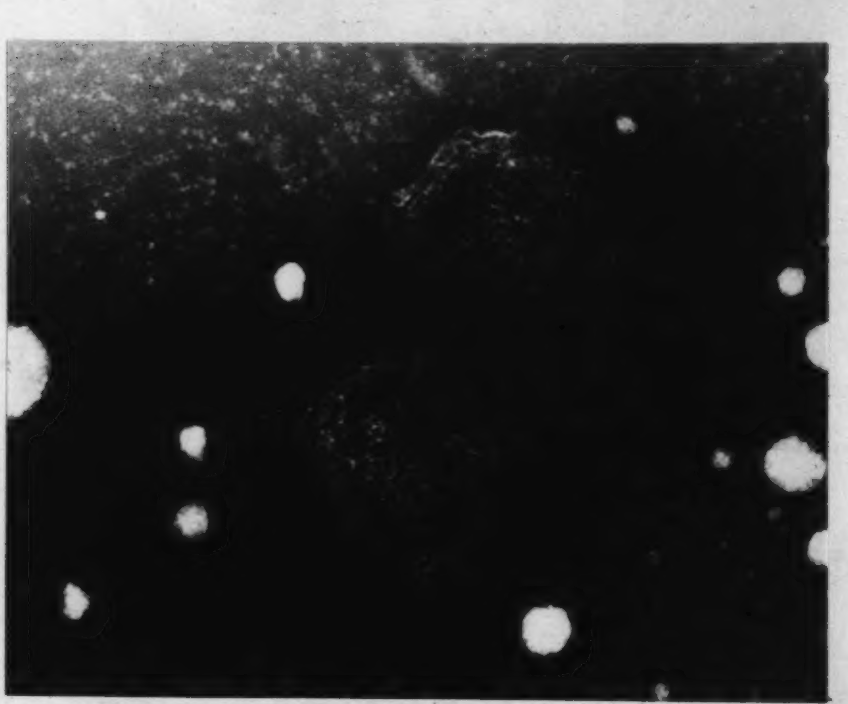
Electron micrograph of a negatively stained preparation of vaccinia virus after treatment with SDC.

Appearance of viral particles after extraction by 0.5% SDC. This photograph shows surface and core alteration. x 137,000.

FIGURE 24

Electron micrograph of a negatively stained preparation of vaccinia virus after treatment with SDC.

A viral particle after extraction by 0.5% SDC showing a smooth surface. x 137,000.



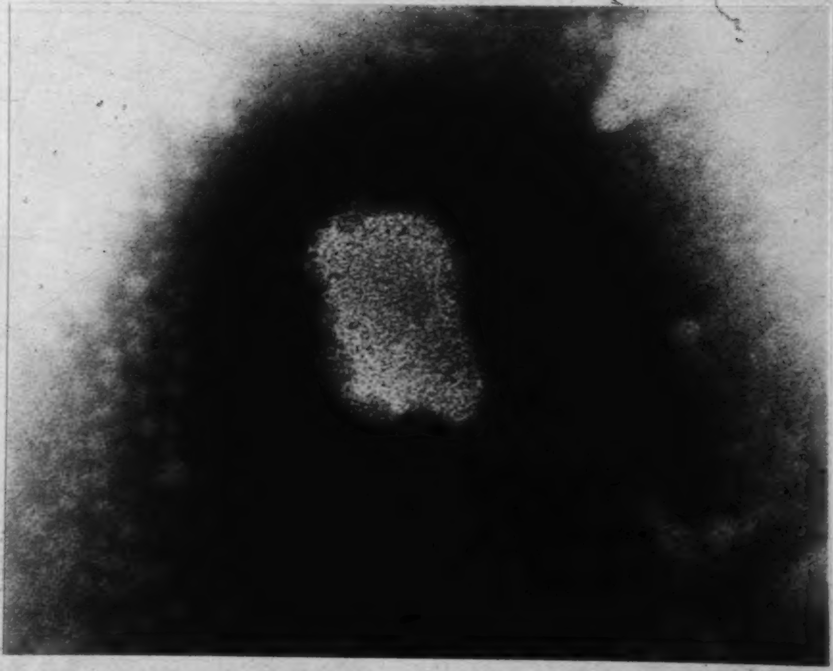


FIGURE 25

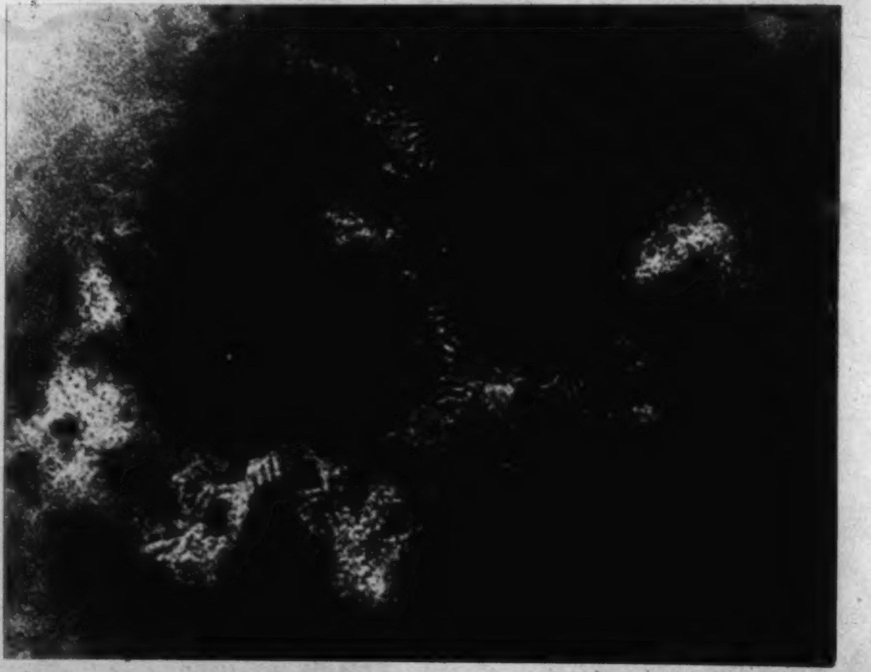
Electron micrograph of vaccinia virus negatively stained after CTAB treatment.

Electron micrograph showing viral material left after treating vaccinia virus with 0.5% CTAB. This photograph shows an aggregate of extracted viral particles with brick-shaped forms at the edges. x 154,000.

FIGURE 26

Electron micrograph of vaccinia virus negatively stained after CTAB treatment.

Higher magnification of the type of aggregates seen in Figure 25, showing the structural organisation within these aggregates. x 245,000.



b) Ultramicrotomy

The technique of ultramicrotomy was used to examine the effects of detergents on the viral ultrastructure, and then to correlate the observations obtained with the results of gel electrophoresis and negative staining.

Figures 27 and 28 show control vaccinia virus cut through at two different planes. A cut perpendicular to the long axis (Fig 27) shows the inner layers of the virus with the core containing the nucleoprotein complex, surrounded by the lateral bodies. The outer layers comprising the viral envelope and the surface protein coat are also seen. Figure 28 is an electron micrograph of a cut parallel to the long axis. It shows a large area of the core, the core envelope, the viral envelope and the surface protein coat which corresponds to the tubules seen by negative contrast staining.

Since the viruses in Figures 27 and 28 were incubated for 3 h at 37°C, freshly purified virus was also examined by electron microscopy to see if this incubation period could have caused any alteration to the viral ultrastructure. Neither negative contrast staining or ultramicrotomy showed any structural differences between the two preparations.

The effects of NP-40 on the viral structure are presented in Figures 29, 30 and 31. Figure 29 is a low magnification view of the treated viruses. The only noticeable effect seems to be associated with the surface protein coat of most viral particles. The outside or edge of the particles is not as smooth as in the control, but has a toothed edge appearance. The arrows are pointing to some particles, showing distinctively this type of alteration.

A higher magnification of the extracted viruses is shown in Figure 30. The toothed edge is readily seen. The outer envelope is still present (arrow) but the surface protein coat has been removed in certain areas. No viruses were seen from which the outer coat had been completely extracted. Apart from the surface coat, no other structural components were detectably affected by the detergent treatment. In a few instances, the core and some of the internal details could be readily seen. Figure 31 shows well preserved particles; the core, the core envelope, the lateral bodies and the viral envelope are still intact. Only the surface coat has been partially removed.

The effects of SDC on the ultrastructure of the virus are shown in Figures 32, 33 and 34. A low magnification view of the extracted particles is presented in Figure 32. Two distinct types of alterations can be observed. First, the edge of the virus is much smoother than the control or NP-40 treated virus. Second, the internal content of the core of some viruses has been changed.

A higher magnification of the extracted viral particles is presented in Figure 33. The surface coat covering the viral envelope has been removed. In some cases, a small amount of material (surface coat) was still remaining on the envelope. The removal of the surface coat gives rise to the sharp edge of the viral particles. As shown in Figure 33, the viral envelope,the lateral bodies and the core envelope appeared intact, but not the inside of the core. Figure 34 shows a view of a virus with an altered core and no surface coat. The nucleoprotein complex which is located inside the core, seems to be shrunken or coagulated. There is much resemblance between the viral particle on the left in Figure 34 and the particles presented in Figure 24. Approximatly 30% of the viral particles appear to have an intact core (see right hand side particle in Figure 34).

The extensive alterations caused by CTAB are presented in Figures 35, 36 and 37. A low magnification examination (Fig 35) showed that the material has aggregated after extraction. The rectangular or brick-shaped appearance of the residual particles can still be recognized. These aggregates seems to be composed of lines which are sometimes parallel (compare to Figs 25 and 26). A closer look reveals that these aggregates are composed of viral particles devoided of their surface coat, their outer envelope and their lateral bodies (arrows).

Figure 36 is a high magnification of one of those particles which shows these parallel lines. This particle has lost most of its structural components. Only the core envelope and some material inside the core is present. Notice the reduction of stained material in the core envelope and inside the core. In this photograph, the internal content of the core has the appearance of tubules organized into an "S" shape. The degree of alteration varied. In some cases, lateral bodies could still be seen attached to the viral cores (less than 10% of the particles examined). Figure 37 shows some particles with their lateral bodies still present (arrows).

Observation of viral particles treated with the detergents and dithiothreitol were made. As for particles examined by negative contrast staining, no morphological differences could be detected, when compared with particles treated with detergents alone.

FIGURE 27

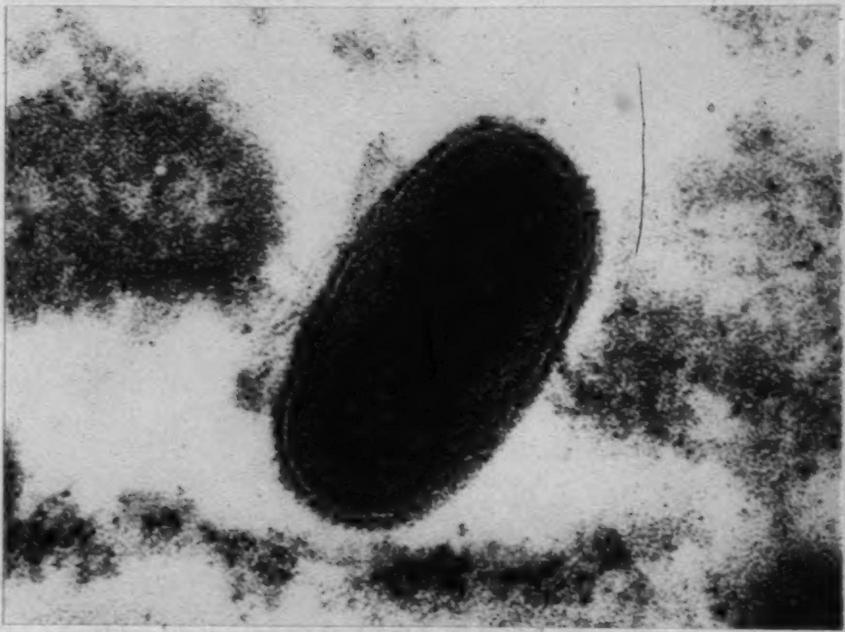
Electron micrograph of a thin section of control (untreated) vaccinia virus.

Thin section of a viral particle incubated for 3 h at 37°C in buffer alone. The sections embedded in Epon 812 were stained with uranyl acetate and lead citrate. This photograph shows the core content, the core envelope, the lateral bodies, the viral envelope and the surface protein coat. x 190,000.

FIGURE 28

Electron micrograph of a thin section of control (untreated) vaccinia virus.

Same as above except that this particle was sectioned through a different plane. This photograph shows the inside of the core envelope, the viral envelope and the surface protein coat. x 190,000.



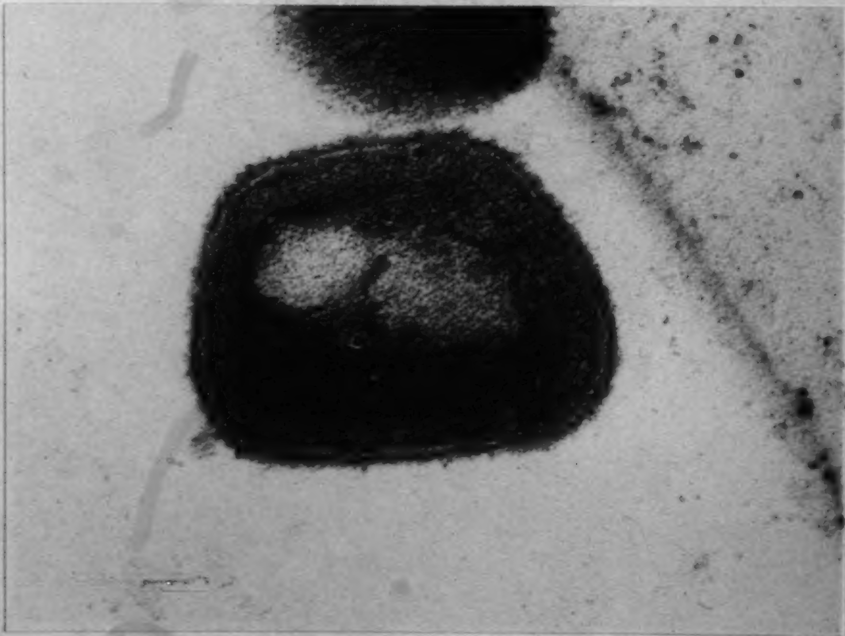


FIGURE 29

Electron micrograph of a thin section of viral particles after NP-40 treatment.

Low magnification view of viral particles after extraction by NP-40. After fixation in glutaraldehyde and osmium tetroxide, the extracted viral particles were embedded in Epon 812. The sections were stained with uranyl acetate and lead citrate. The arrows point to viral particles showing clearly their toothed edges. x 28,000.

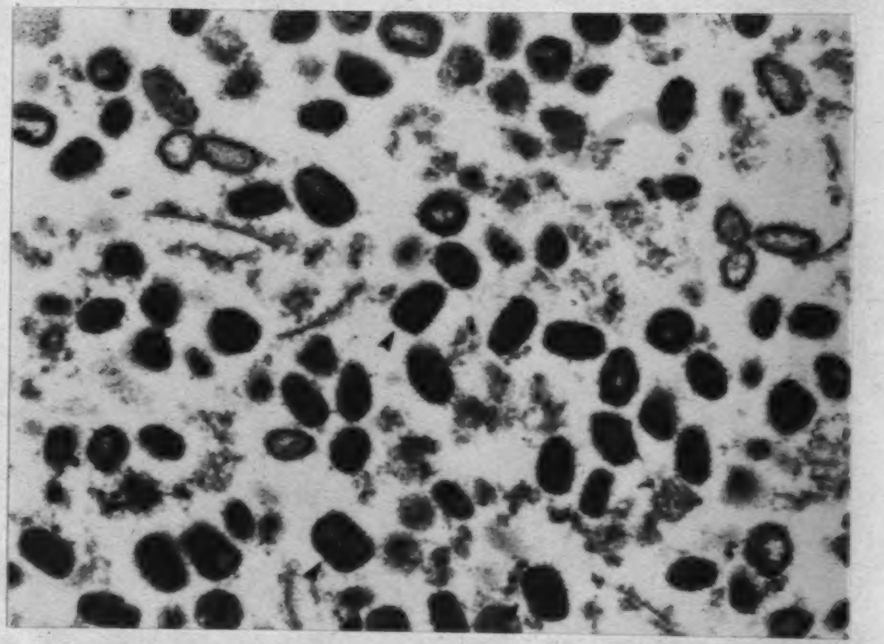


FIGURE 30

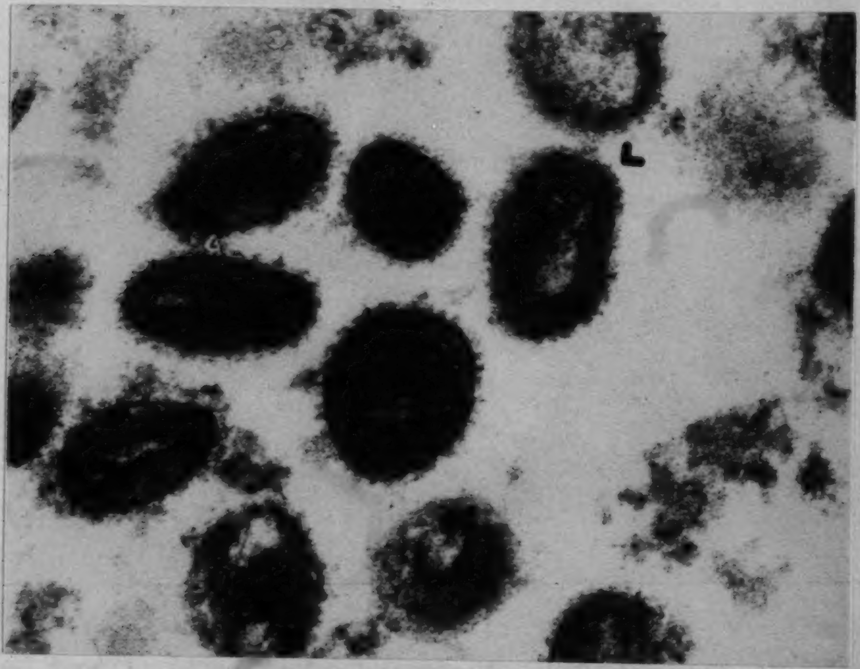
Electron micrograph of a thin section of viral particles after NP-40 treatment.

High magnification of viral particles after extraction by NP-40, showing the intact viral envelope and the partially removed surface protein coat. x 91,000.

FIGURE 31

Electron micrograph of a thin section of viral particles after NP-40 treatment.

High magnification of viral particles after extraction by NP-40 showing a virus with the inside of the core, the core envelope, the lateral bodies and the viral outer envelope not detectably affected by NP-40. x 161,000.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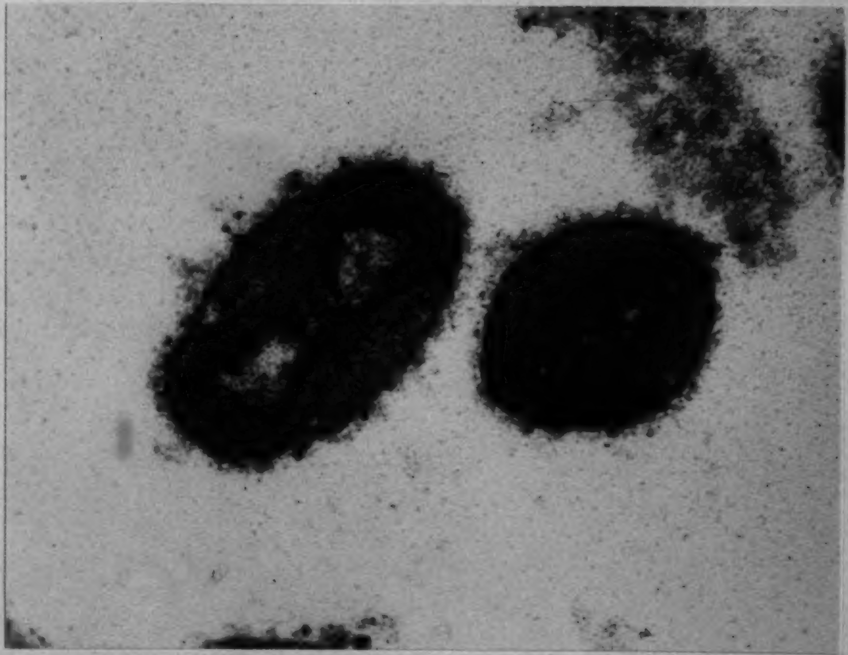


FIGURE 32

Electron micrograph of a thin section of vaccinia virus after SDC treatment.

Low magnification view of viral particles extracted by SDC. Notice the altered core of some of the viral particles. x 28,000.

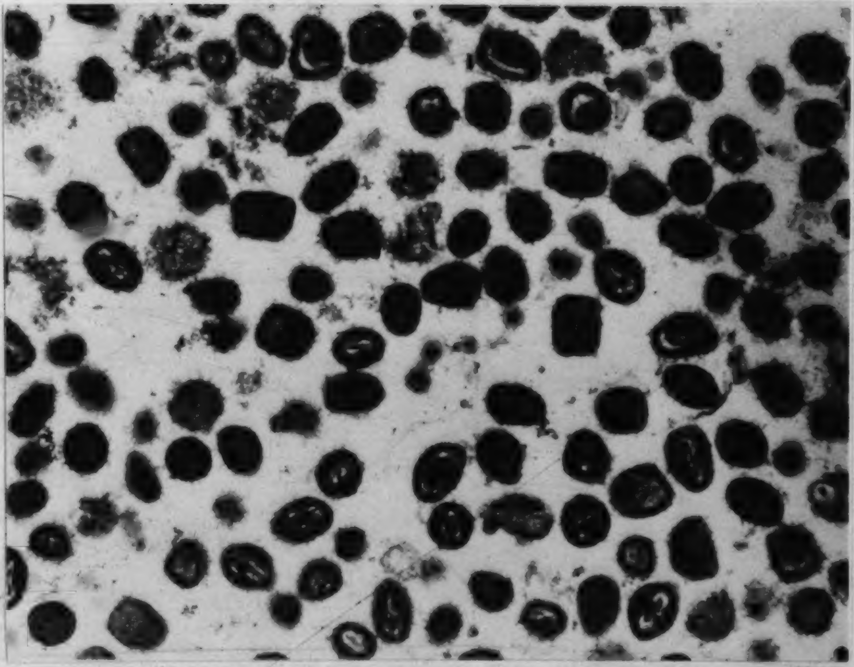


FIGURE 33

Electron micrograph of a thin section of vaccinia virus after SDC treatment.

High magnification view of viral particles after extraction by SDC. The surface coat covering the envelope is removed but the envelope itself appears intact. Both particles have an altered core. x 129,000.

FIGURE 34

Electron micrograph of a thin section of vaccinia virus after SDC treatment.

High magnification view of viral particles after extraction by SDC. Notice the appearance of the core in the particle on the left, as compared with the one of the particle on the right. x 135,000.

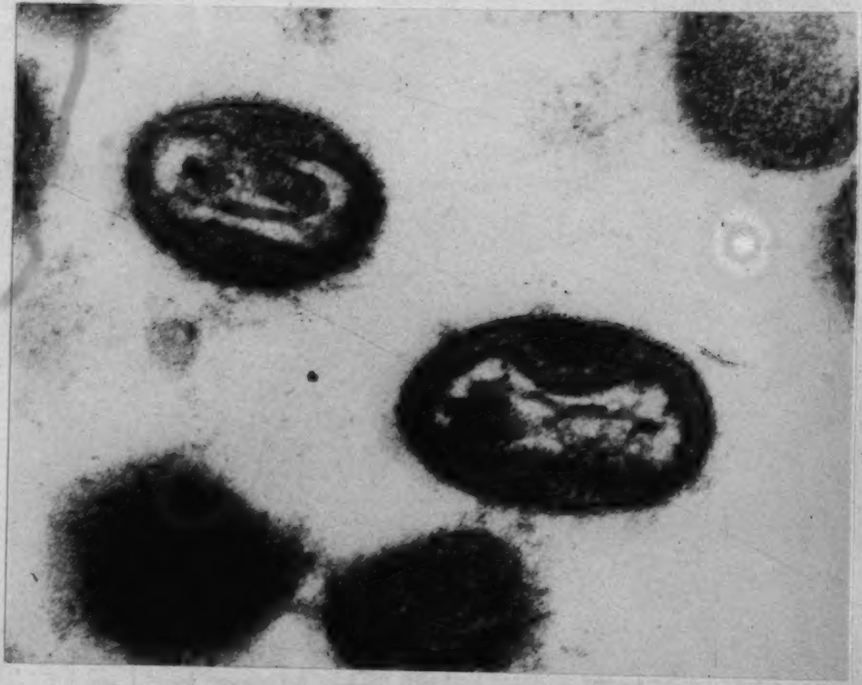


FIGURE 35

Electron micrograph of a thin section of residual viral particles after CTAB treatment.

Low magnification view of the viral material left after extraction by 0.5% CTAB. Notice the absence of electron dense material associated with the residual particles. The arrows point to particles showing a core with a tubular appearance. x 54,000.

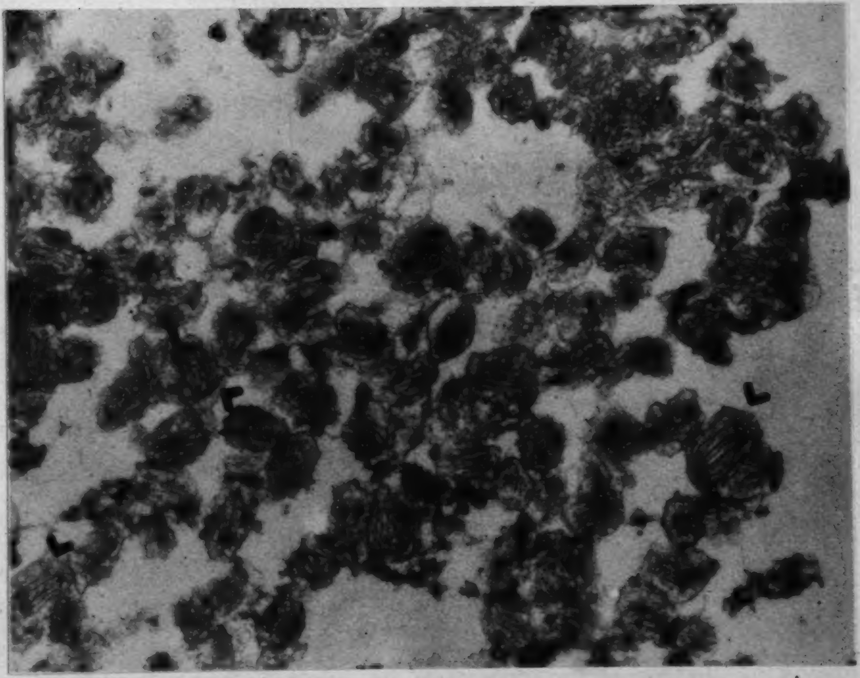


FIGURE 36

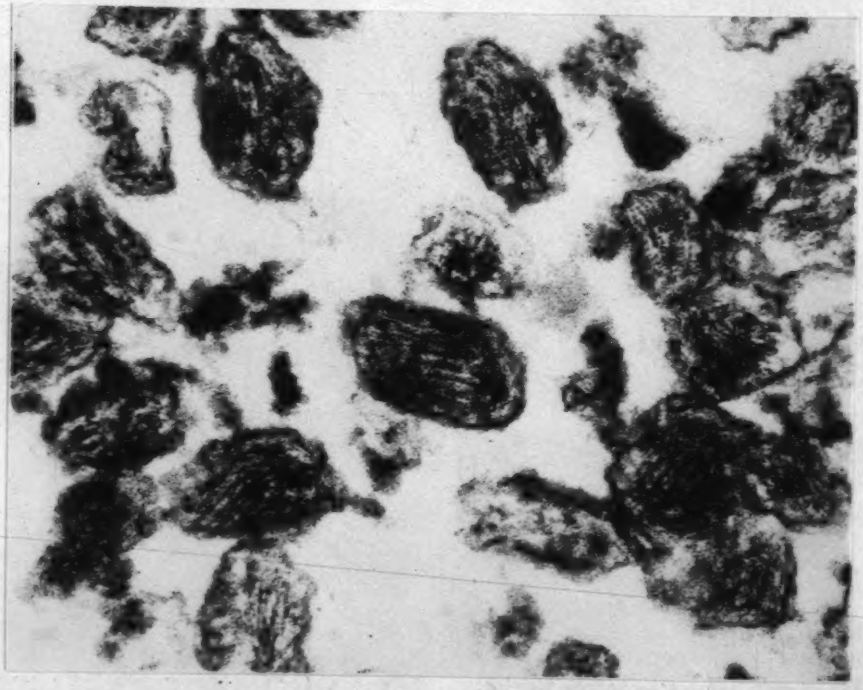
Electron micrograph of a thin section of residual virus after CTAB treatment.

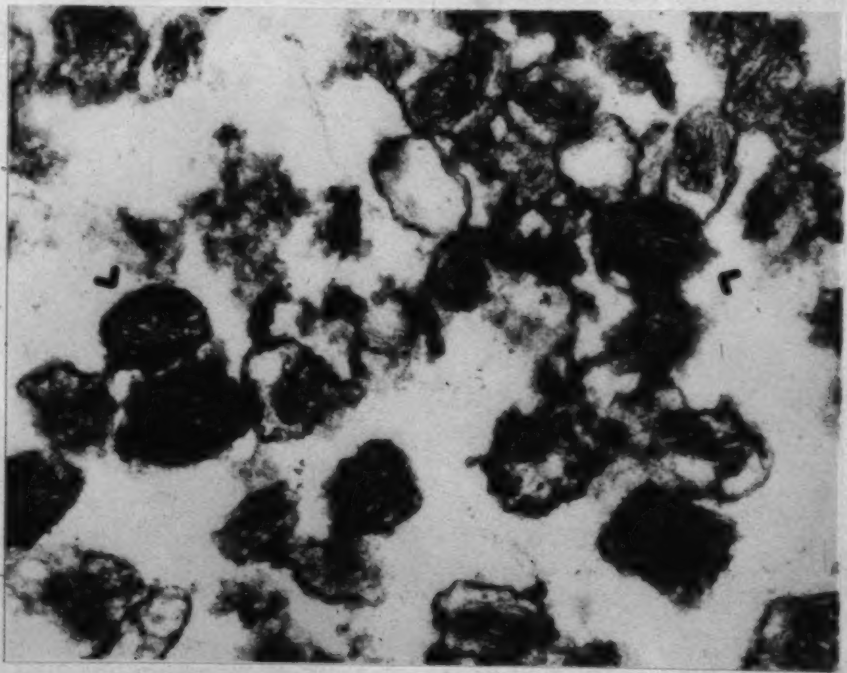
High magnification of residual viral particles after extraction by CTAB. In the center of the photograph, is an example of a particle showing the "S" shape appearance of the inside of the core. x 100,000.

FIGURE 37

Electron micrograph of a thin section of residual virus after CTAB treatment.

High mignification view of residual viral particles after extraction by CTAB. The arrows point to particles still covered by their lateral bodies. x 68,000.





PURIFICATION OF COMPLEX E FROM AN NP-40 EXTRACT

It was concluded from the results presented earlier that among the detergents tried, NP-40 was the most appropriate to extract antigenic material from the virions. Although chemical means of extracting proteins have an advantage in that one does not have to cope with the removal of detergents, such methods were not satisfactory since they release less than 10% of the total viral protein content.

When NP-40 was used to extract vaccinia virus, one major antigen (determined by microimmunodiffusion) was solubilized. This NP-40 extract accounted for about 20% of the total viral protein content. This extract analysed by SDS gel electrophoresis was found to contain 11 polypeptide bands of which VP-6a and VP-6b were the most intensely stained bands.

Unlike the ionic detergents, NP-40 did not seem to affect the immunological properties of the extracted material nor did dialysis of the extract precipitate the solubilized material. Hence, it was decided to utilize NP-40 to extract vaccinia virus, and to use this extract for further purification of the major precipitin band.

1) Extraction of vaccinia virus by NP-40

Purified virus was dialyzed overnight at 4°C against 0.05M phosphate buffer pH 7.4. The next day, the viral suspension was diluted to 1 mg of viral protein per ml of buffer and NP-40 was added until a final concentration of 0.5% (w/w) was achieved. This mixture was incubated for 3 h at 37°C on a small rotatory shaker. After incubation, the viral suspension was centrifuged at 40,000xg for 90 min. The clear supernatant was dialysed overnight at 4°C against 0.05M phosphate buffer pH 7.4. A total of 7.8 mg of viral protein in 45 ml was extracted from 45 mg of purified virus. This corresponds to 17% of the total viral protein content. The extract examined by microimmunodiffusion was found to have a gel pattern identical to that reported earlier (Fig 15).

2) Gel chromatography of the NP-40 extract

The sample (7.5 mg of protein), was concentrated at 4°C by dialysis against polyethylene glycol (PEG 20,000). The sample concentrated to 2.8 ml was then loaded onto a G-200 colum (2.5 x 35 cm) and eluted with 0.05M phosphate buffer pH 7.4. Fractions of 2 ml were collected. The presence of detergent in the sample interfered greatly with the collection of fractions by decreasing the fraction volume to less than 1 ml because of the reduced surface tension of the drops. Not until the internal volume of the column was reached did the fraction volume return to 2 ml.

The fractions collected were analysed by the Lowry procedure for protein concentration and by absorbance at 280 nm. Since NP-40 has an ultra-violet absorption spectrum similar to that of protein and an extinction coefficient greater than that of protein at 280 nm, the absorbance at 280 nm of the collected fractions indicates more the distribution of the detergent rather than the protein. The elution profile is presented in Figure 38. The profile monitered at 280 nm is composed of a peak eluting at the void volume (Vo) followed closely by a second peak, and finally a third peak appearing with the internal volume of the elution column (Vi). The void volume peak contained mainly NP-40 micelles. The second peak following the void volume is due to the complex E. This second peak contains both protein and detergent as indicated in Figure 38. The last peak (Vi) in Figure 38 contained both protein and detergent. The material present would have a small size since it represent the lowest size limit of resolution of the gel (less than 5,000 daltons). This last peak must be some kind of artifact since neither disc gel electrophoresis or SDS gel electrophoresis gave any stainable band. This peak was not characterized further.

3) Composition of complex E peak

Fractions on either side of the complex E peak were pooled and analysed by various means to find how many components were present in the peak. The fractions selected for analysis were 38, 39, 41, 54, 55, 56, 65, 66 and 67.

Analysis of the fractions was done by microimmunodiffusion, immunoelectrophoresis, analytical ultracentrifugation, disc gel electrophoresis and SDS gel electrophoresis as described in Materials and Methods.

The microimmunodiffusion results are shown in Figure 39. Because some of the samples precipitated at room temperature, the diffusion was carried out at 4°C. The plate, after 5 days of incubation, showed a single band of precipitation between vaccinia virus antiserum and the wells containing the various fractions. No precipitin reaction was observed when host cell or pre-immune serum were used.

The result obtained after immunoelectrophoresis is shown in Figure 40. The photograph taken after 5 days of incubation, shows a single precipitin band when a sample from the complex E peak was placed in both wells and separated by electrophoresis. The results also indicate that the component reacting with the viral antiserum did not have any electrophoretic mobility at pH 8.4.

Figure 41 shows the results obtained when a sample was analysed by analytical ultracentrifugation. A single symmetrical peak was present. When the Schlieren pattern was examined later during centrifugation, a single peak was still observed but the trailing edge of the peak was slightly skewed. Microheterogeneity of complex E or the presence of the detergent could cause such skewing. When fractions containing less detergent were centrifuged (such as fraction 66 pooled), the peak remained symmetrical throughout the run. When fractions containing more detergent (such as fraction 39 pooled) were centrifuged the skewing of the trailing edge of the peak was more pronounced.

The samples were also analysed by discontinuous disc gel electrophoresis. The results obtained are presented in Figure 42. A large band of protein (double arrow) was found around the top of the gel but a small stained band (single arrow) migrating slightly faster was also present. Since 200 µg of protein were put on the gel, this small band would represent a small percentage of the total amount of protein.

Samples were analysed by SDS gel electrophoresis. The results are shown in Figure 43. Fraction 55 (pooled) was found to contain a total of 11 polypeptide bands of which the VP-6 group of polypeptides and VP-10 were the most abundant. The polypeptide VP-10 was the second most abundant polypeptide.

FIGURE 38

Elution profile from Sephadex G-200 of an NP-40 extract of vaccinia virus.

An NP-40 extract from vaccinia virus was dialysed, concentrated and chromatographed on Sephadex G-200. The fractions obtained were assayed for protein by the Lowry procedure and by absorbance at 280 nm. The packing of the column and the void volume were determined with the use of Blue Dextran 2000.

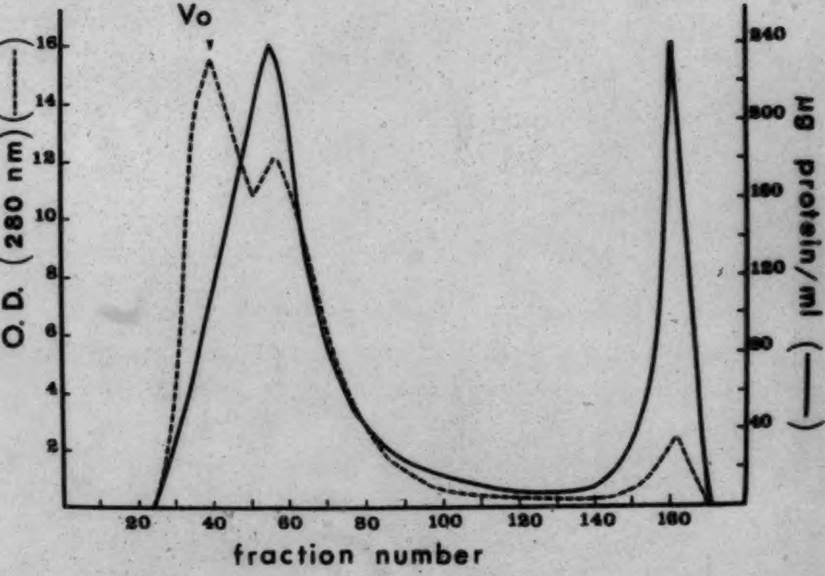

Vo
O.D. (280 nm) (------)
16
14
12
10
8
6
4
2
μg protein/ml (——)
240
200
160
120
80
40
20
40
60
80
100
120
140
160
fraction number

FIGURE 39

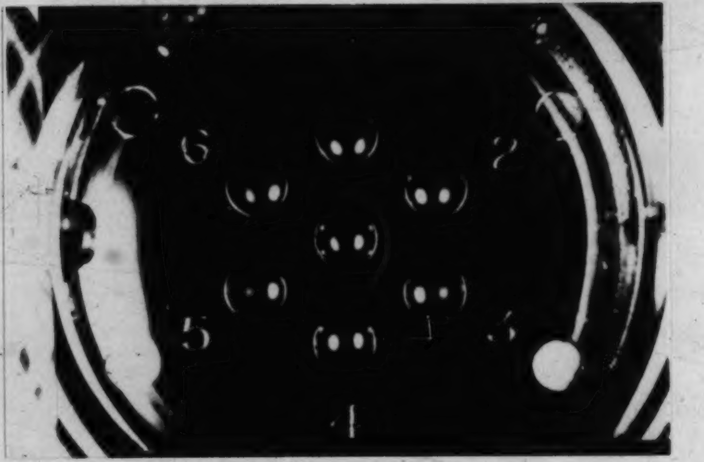
Microimmunodiffusion assay of complex E peak.

Fractions of the complex E peak were analysed by microimmunodiffusion. The center well contains viral antiserum (undiluted). Wells 1, 2 and 3 contain fractions 39 (pooled), 55 (pooled) and 66 (pooled), and wells 4, 5 and 6 contain PBS. The photograph was taken after 5 days of incubation.

FIGURE 40

Immunoelectrophoresis analysis of complex E peak.

Sample 55 (pooled) was analysed by immunoelectrophoresis. The middle trough contains viral antiserum (undiluted) and both wells had received the antigen solution. The photograph was taken after 5 days of incubation.


6
2
5
4
3
1

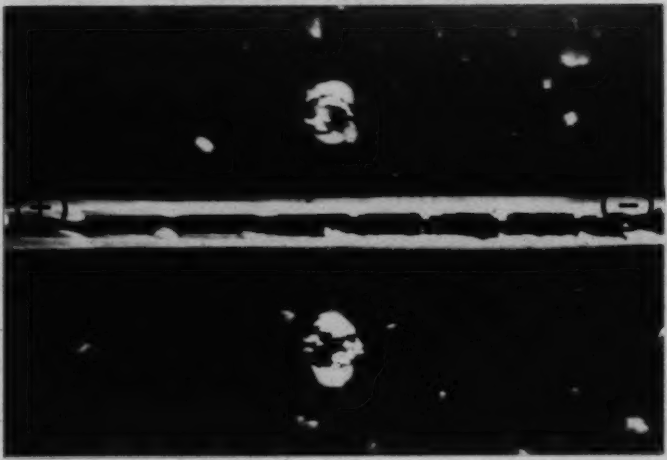

+
−

FIGURE 41

Ultracentrifugation analysis of complex E

Fraction 55 (pooled) was analysed by analytical ultracentrifugation as described in Materials and Methods. The top photograph was taken 64 min after the rotor had reached a speed of 60,000 rpm. The bottom one was taken after 124 min. Sedimentation is from left to right.

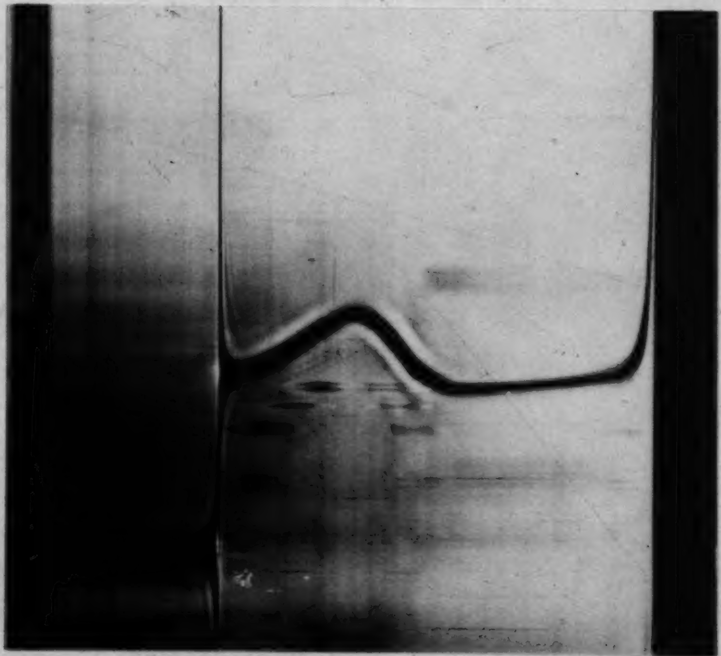


FIGURE 42

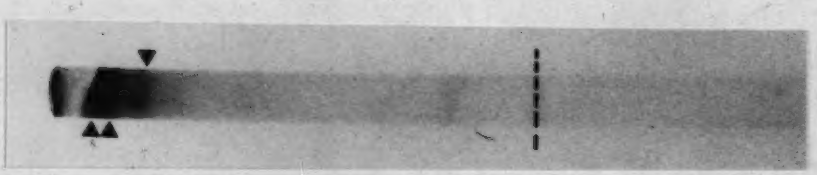
Gel containing complex E analysed by discontinuous disc gel electrophoresis.

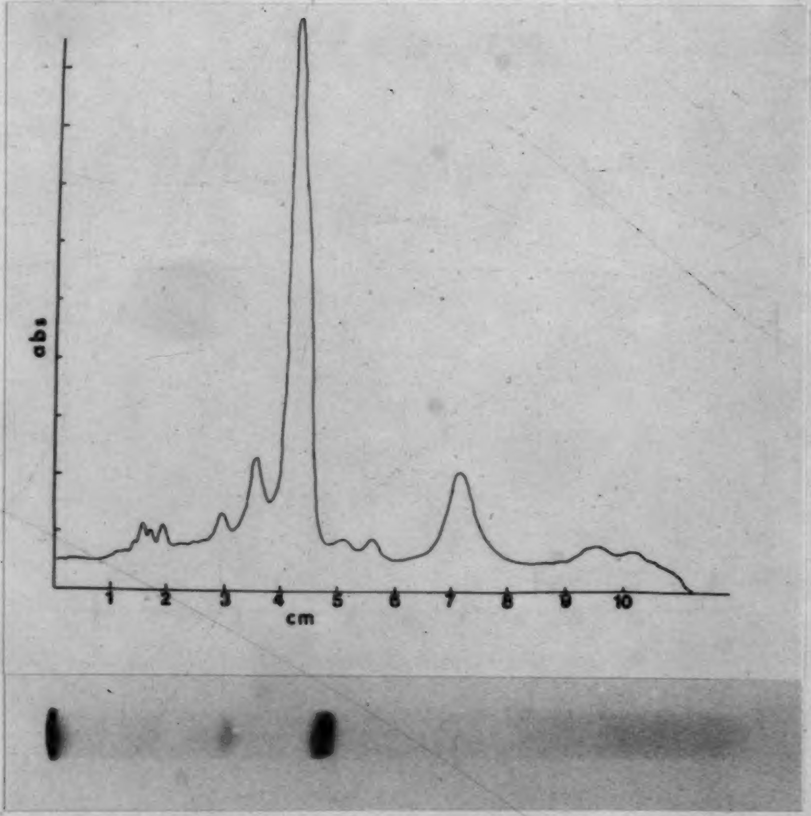
Analysis of sample 55 (pooled) by disc gel electrophoresis. A total of 200 μg of viral protein was electrophorezed. The arrow (single) points to a possible contaminant. The marker dye is indicated by a dotted line. The direction of electrophoresis is from left to right.

FIGURE 43

Densitometer tracing of an SDS gel containing a sample from the complex E peak.

A total of 100 μg of viral protein from sample 55 (pooled) was solubilized in SDS as described in materials and methods and analysed by SDS gel electrophoresis. The direction of electrophoresis is from left to right.




abs
1
2
3
4
5
6
7
8
9
10
cm

PRODUCTION OF ANTISERUM AGAINST COMPLEX E

Antisera were produced against complex E as described in Materials and Methods. Fractions 30-74 were utilized for the production of antibodies. The activity of the antisera obtained was analysed by microimmunodiffusion against a fresh NP-40 extract and a soluble viral antigen fraction obtained by high speed centrifugation of sonicated infected cells.

The results are presented in Figures 44, 45 and 46. Figure 44 indicates that a single precipitin band was found against all the serum obtained. This figure also shows a small ring of non-specific precipitation around the atigen well, caused by the detergent NP-40. Figure 45 shows the results obtained when a soluble viral antigen solution was used as the only source of complex E, free of detergent. A single precipitin band was also obtained. Since this soluble antigen fraction contains only a small amount of complex E, only the strongest antisera reacted (well #3). The other serum having a lower antibody titer did not produce a precipitin band until the antigen solution was highly concentrated. Figure 46 shows the results obtained when the soluble viral antigen fraction and the fresh NP-40 extract were tested for antigenic identity. Because of the large difference in the concentration of complex E and in the presence of NP-40 in one of the antigen solutions, the system was difficult to balance. The precipitin bands never formed a strong identity reaction but no spur formation could be detected.

Earlier evidence (electron microscopy) had pointed out the possibility that the material extracted by NP-40 was located on the surface of the viral particles. If antibodies against complex E could neutralize the virus, it would be indicative of the presence of complex E, accessible on the surface of vaccinia virus.

A neutralization assay was carried out as described in Materials and Methods. All the sera tested (7 of 7) were found to contain neutralizing antibodies. The strongest antiserum (determined by immunodiffusion) was found to have a 50% plaque reduction titer of 12,000. It is concluded that antibodies against complex E can effectively neutralize vaccinia virus.

The reduction in the plaque count was found only when specific antisera were used. Control sera (described in Materials and Methods) did not have neutralizing activity when assayed by the plaque reduction assay.

FIGURE 44

Antiserum obtained against complex E, analysed by immunodiffusion against an NP-40 extract.

The center well contains a fresh NP-40 extract. Well #1 contains pre-immune sera and the other wells contain the antiserum obtained from various rabbits.

FIGURE 45

Antiserum obtained against complex E, analysed by immunodiffusion against a soluble viral antigen solution.

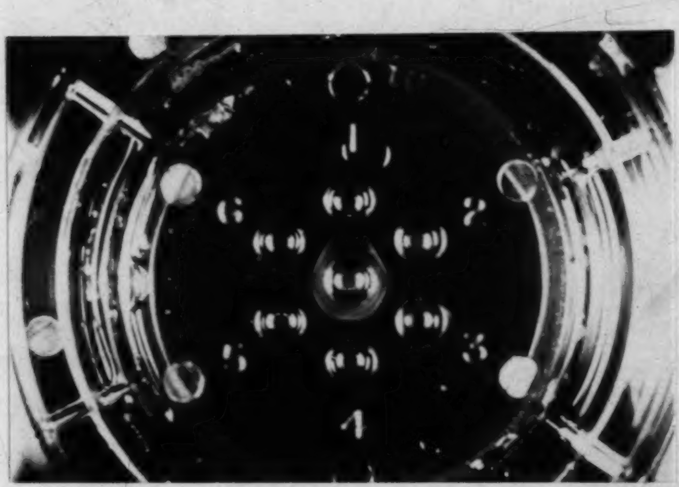
Similar to Fig 44 except that the center well contains the soluble viral antigen solution. Only the strongest antisera #3 gave a precipitin band.

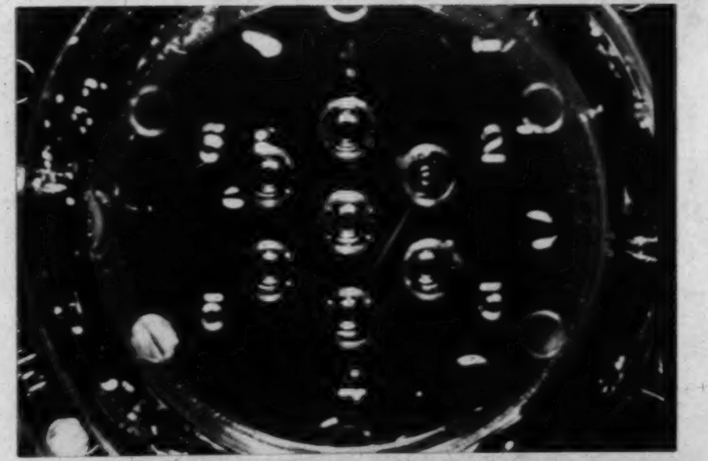
FIGURE 46

Antiserum obtained against complex E, analysed by immunodiffusion against extracts containing complex E.

The top wells contain antiserum against complex E. The bottom wells contain: a) fresh NP-40 extract, and b) soluble viral antigen fraction. Non-specific bands of precipitation are seen around the well containing the NP-40 extract.

All the sera used in the above figure were undiluted. The photographs were taken after 5 days of incubation.


1
2
3
4
5
6


5
2

LOCALIZATION OF COMPLEX E IN INFECTED CELLS, BY THE IMMUNOFERRITIN TECHNIQUE

Since electron microscopy and neutralization experiments suggested that the complex E molecule extracted by NP-40 was located on the surface of vaccinia virus, a study using the immunoferritin technique was performed to confirm the location of complex E.

1) Effect of fixation on the morphology of LLC-MK2 cells.

Cells were infected and fixed 24 h later in glutaraldehyde or paraformaldehyde as described in Materials and Methods. After fixation at 4°C, the cells were embedded in GMA as described in Materials and Methods. Sections were cut, stained with uranyl acetate and lead citrate and examined by electron microscopy to determine the quality of the fixation procedures. All the various concentrations of fixatives and times of fixation were similar in preserving the morphological features of the cell and virus. A 30 min fixation by a 1% solution of glutaraldehyde preserved the morphology of the cell as well as a 4 h fixation in a solution of 5% paraformaldehyde. At no time was osmium tetroxide used as a fixative, because of its denaturing effect.

Most morphological components of the cell as judged by the appearance of the nucleoli, the nuclear membrane and the mitochondria are well preserved (Fig 47). A certain amount of material has been extracted from the cell. This extraction is characterized by the appearance of empty areas in the ground cytoplasm and especially around the viral particles.

Figure 48 and 49 are high magnification photographs of a cell in which the mitochondria, the nuclear membrane, the cell membrane, and the viral particles are well preserved. These two figures (48 and 49) show particularly well the good preservation of the membranes which is considered as a criterion of fixation.

2) Immunoferritin staining of thin sections.

Staining of the infected cells by the indirect immunoferritin technique has been described in materials and methods (see Figure 2). The blocks containing the cells fixed in 1% glutaraldehyde for 30 min and in 2.5% paraformaldehyde for 30 min were used for staining.

Four different kinds of controls were tested:

a) infected cells and ferritin conjugate.
b) infected cells, non-specific antiserum (anti-adenovirus type 2) and specific ferritin conjugate
c) infected cells, specific antiserum and non-specific ferritin conjugate (anti-human gamma-globulin, sheep gamma-globulin was conjugated to ferritin).
d) non-infected cells, specific antiserum and specific ferritin conjugate.

Conditioning the sections in 4% BSA was found to reduce the background, especially the non-specific adsorption of the conjugate to the embedding medium and to the

Formvar. The use of whole serum diluted with PBS as compared to the use of purified antibodies did not significantly increase the background level. Neither did the concentration of the ferritin conjugate when the concentration was varied between 0.5 to 1.5 mg/ml.

Some of the controls are presented in Figures 50, 51, 52 and 53. These controls were treated with the specific conjugate only (control a) since all the others gave comparable level of background.

A low background level was found on the Formvar itself (Fig. 50). The ferritin background was found to be higher over the GMA embedding medium (Fig. 51). The amount of ferritin background found in the cytoplasm of an infected cell (Fig 52) and the amount of ferritin grains non-specifically attached to viral particles in the cytoplasm (Fig 53) were about the same. Since the level of background over the GMA was similar to that found over the cytoplasm and the viral particles, it would indicate that the embedding medium was mainly responsible for the non-specific attachment of the ferritin conjugate.

None of the last three figures (51, 52 and 53) were stained with uranyl acetate so that the small grain density found in the controls would not be masked by the stain.

When sections were stained with specific antiserum and specific ferritin conjugate, the optimum conditions were as follows: antiserum diluted 1:5 with PBS and the ferritin conjugate at a concentration of 1.0 to 1.5 mg/ml. Staining was accomplished in 2% uranyl acetate in water for 20 min.

Ferritin grains were always found associated with the outer layer (envelope and surface coat) of the viral particles as shown by the Figures 54, 55 and 56. Figure 54 shows viral particles with ferritin grains located over the outer layer. Both mature and immature viral particles had ferritin grains located over their outer layer. Figure 55 shows a typical dumbell shape virus covered with ferritin grains. Figure 56 shows an immature and a mature form of vaccinia virus with ferritin grains located over their outer layer.

Ferritin grains were not found in large concentration over the lateral bodies or over the cores. Figures 54 and 55 shows that the complex E was not present in the core of the virus.

Ferritin grains were present in the cytoplasm of the infected cells but because the cytoplasm might have shrunk or might have been extracted during the fixation and embedding procedures, no grain density was calculated.

A low magnification view of an infected cell is shown in Figure 57. Figures 58 and 59 are enlarged areas of Figure 57. These photographs shows clearly the grain distribution over the outer layer of the immature and mature forms of the viral particles. They also show that the particles were not all labelled to the same extent.

3) Ferritin grain distribution in infected cells.

To find the average number of ferritin grains over the viral particles, negatives of electron micrographs were examined and only grains directly situated over the viral particles (mature) were counted. As a control the same type of count was performed using infected cells stained only with the ferritin conjugate.

For background counts, negatives of electron micrographs of Formvar of GMA were used. From those negatives, the number of ferritin grains per square micron (μ^2) could be determined. To give a better idea of the background over the viral particles, the counts will be expressed per 0.09 μ^2. The latter figure represent an approximation of the surface occupied by a section of a mature viral particle in a thin section. Since the number of grains is expressed per viral particles, the expression of grains per 0.09 μ^2 will be equivalent. The results of the grain distribution are presented in Table 5.

The average number of grains over the viral particles when specific antiserum is used, is 8 times larger than with the controls. Table 5 also shows that most of the background count is caused by the non-specific attachment of the conjugate to the embedding medium. The background count due to the Formvar is almost negligeable.

The distribution of ferritin grains is presented in Figure 60. There an obvious difference in the grain count when specific antiserum is used. About 6% overlapping was found.

FIGURE 47

Thin section of a monkey kidney cell (LLC-MK2), infected with vaccinia virus.

Infected cells were fixed in 2.5% paraformaldehyde for 30 min at 4°C and embedded in GMA as described in Materials and Methods. The section was stained with uranyl acetate and lead citrate.

no: nucleoli, n: nucleus, nm: nuclear membrane, mc: mitochondria, m: mature viral particles, i: immature viral particles. x 12,900.

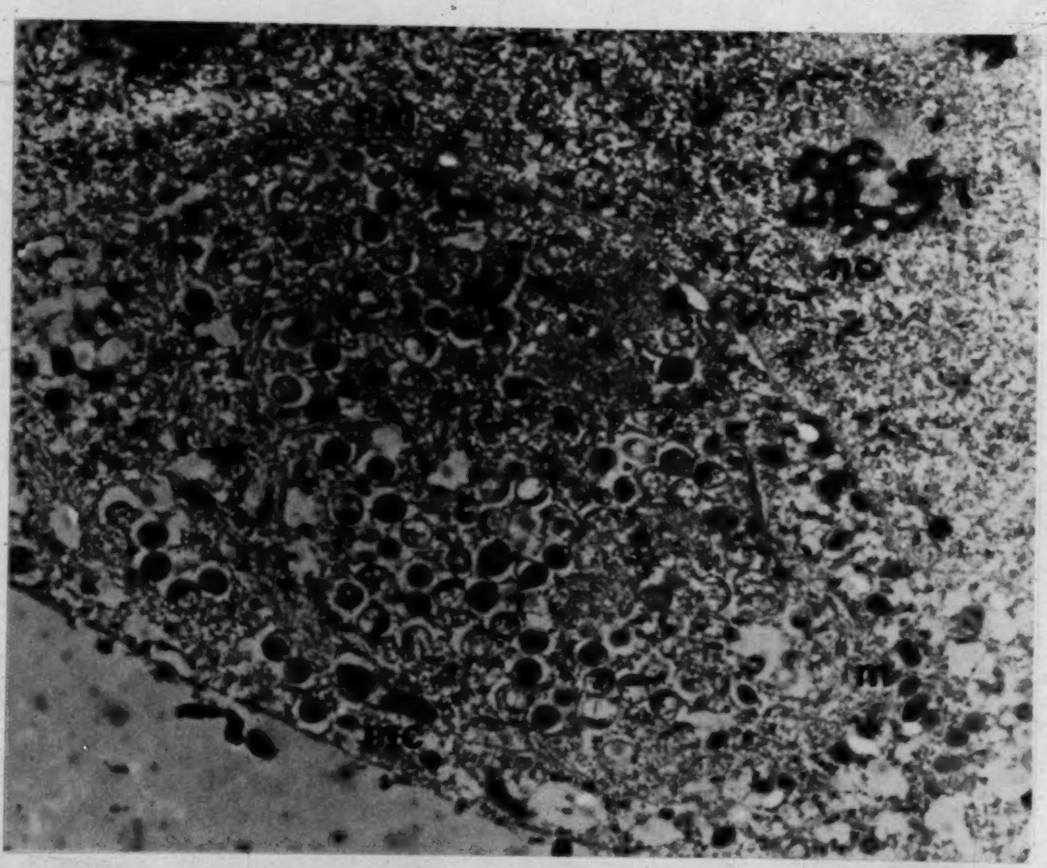

no
m
mc

FIGURE 48

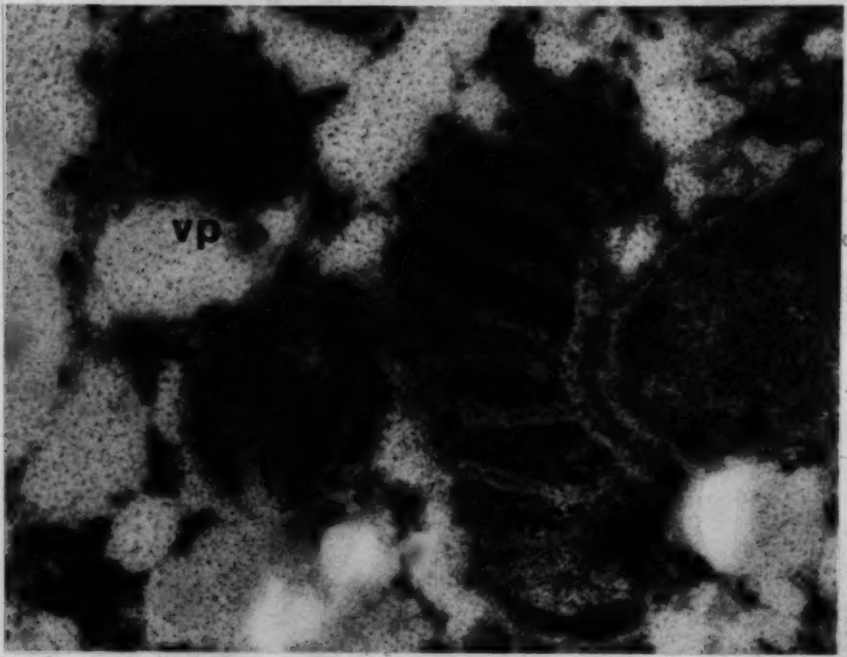
Thin section of a LLC-MK2 infected cell

Thin section of an infected LLC-MK2 cell, fixed in 2.5% glutaraldehyde for 30 min at 4°C. vp: viral particle, mc: mitochondria . x 108,500.

FIGURE 49

Thin section of LLC-MK2 infected cell

Thin section of an infected LLC-MK2 cell, fixed in 2.5% glutaraldehyde for 30 min at 4°C. cm: cell membrane, nm: nuclear membrane, vp: viral particle. x 104,500.


vp

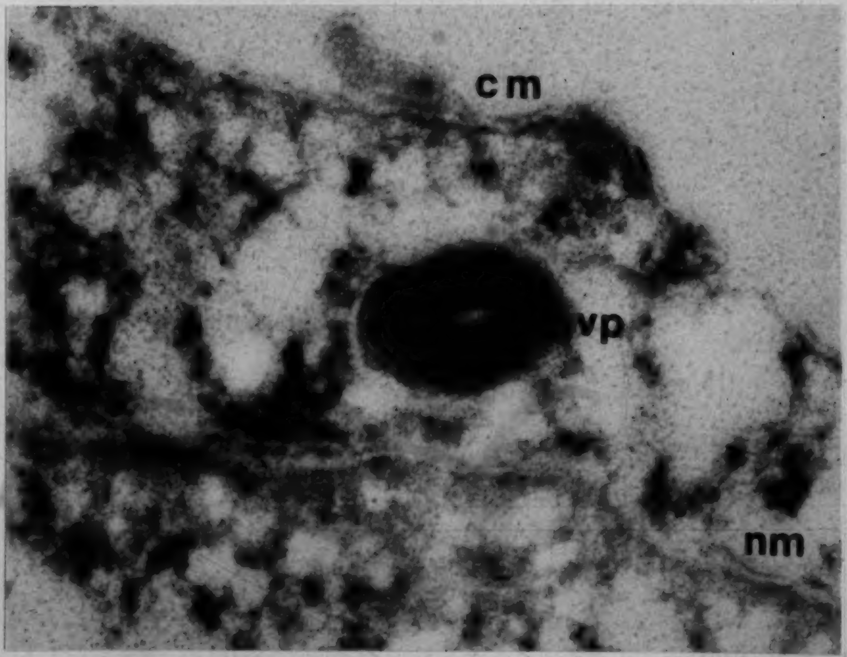

cm
vp
nm

FIGURE 50

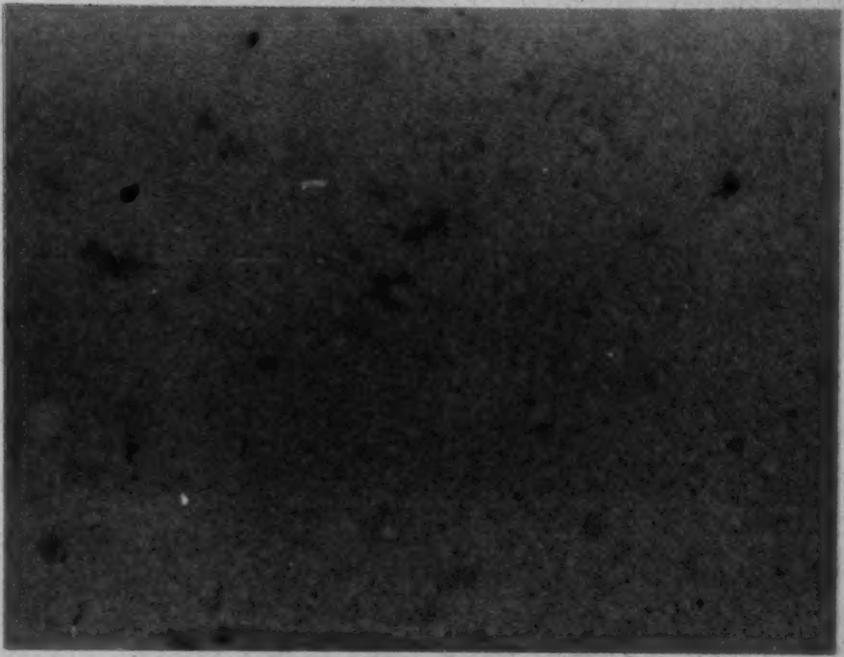
Non-specific ferritin grain background over a Formvar film

Ferritin grain background on a Formvar film stained with specific conjugate. x 79,500.

FIGURE 51

Non-specific ferritin grain background over the embedding medium

Ferritin grain background over the embedding medium (GMA), stained with specific conjugate. x 75,000.



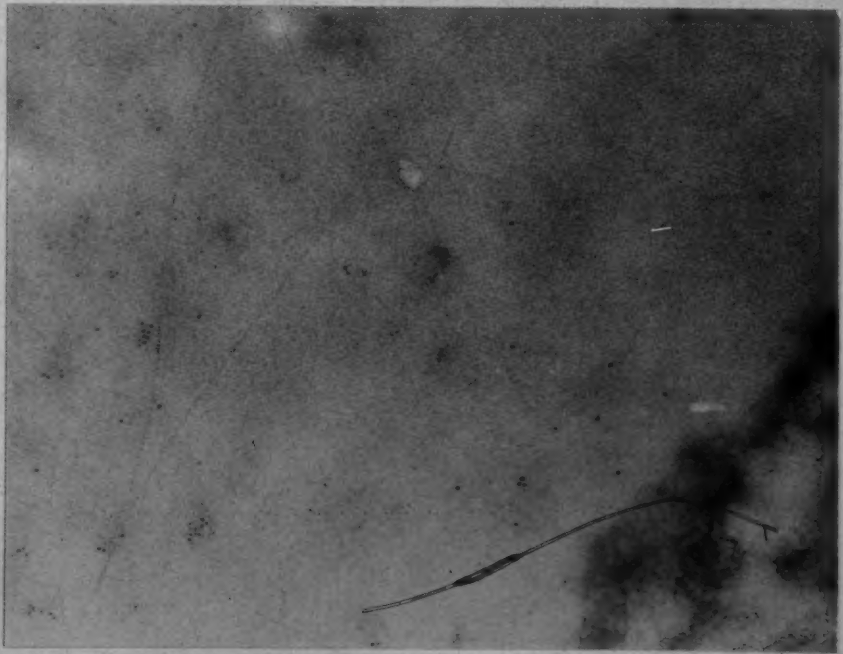


FIGURE 52

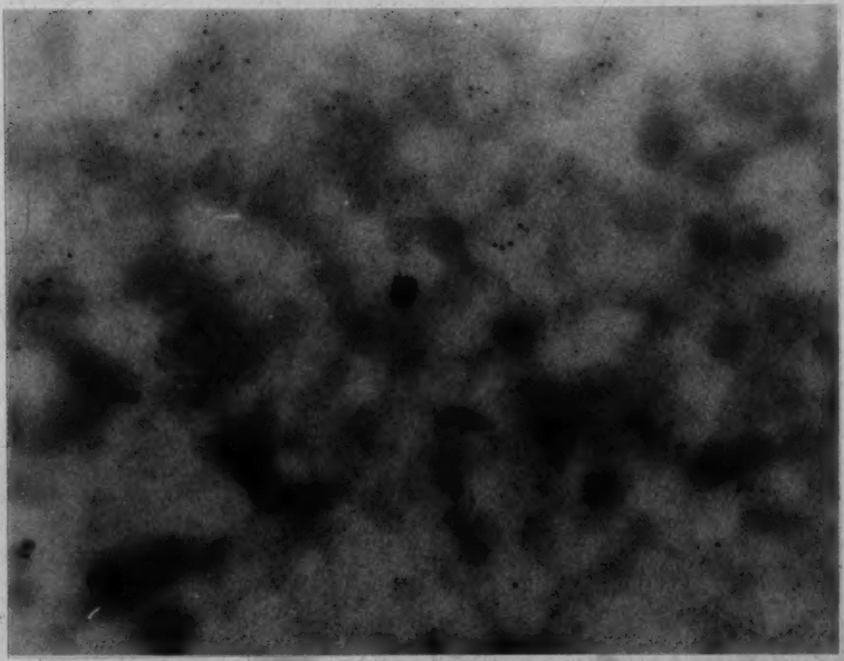
Non-specific ferritin grain background over the cytoplasm

Non-specific ferritin grain background in the cytoplasm of an infected cell. This thin section was labelled with ferritin conjugate only, and was not stained with uranyl acetate. x 97,000.

FIGURE 53

Non-specific ferritin grain background over viral particles

Non-specific ferritin grain background over viral particles, in the cytoplasm of a LLC-MK2 cell. This thin section was labelled with specific conjugate only, and not stained with uranyl acetate. x 73,500.



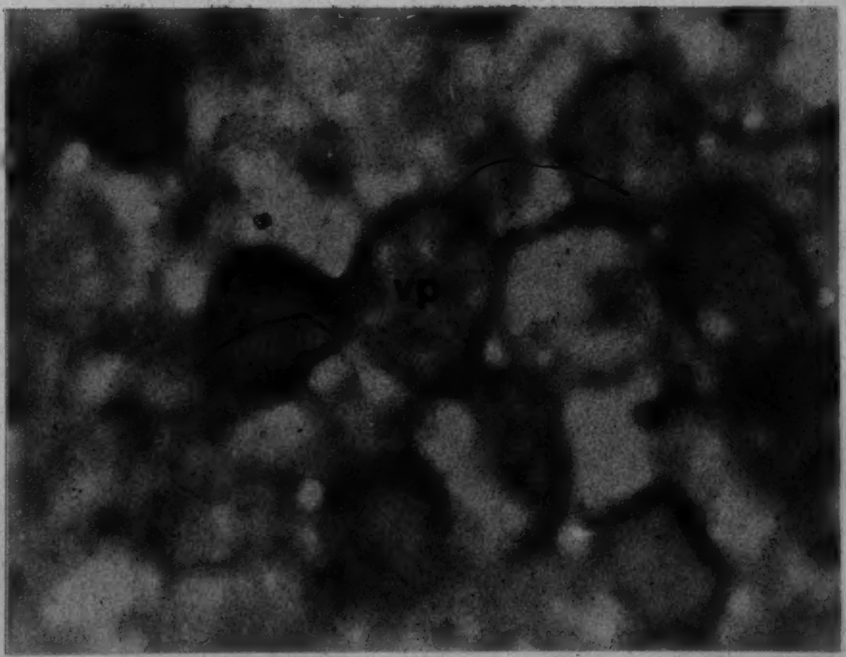

vp

FIGURE 54

Thin section of an infected cell showing viral particles stained by the indirect immunoferritin technique using anti-complex E antiserum.

Vaccinia virus particles in the cytoplasm. The cells were fixed in 2.5% paraformaldehyde for 30 min. x 98,500.

FIGURE 55

Thin section of an infected cell showing viral particles stained by the indirect immunoferritin technique using anti-complex E antiserum.

A viral particle showing excellent preservation of the nucleoprotein complex. The cells were fixed in 1% glutaraldehyde for 30 min. x 173,000

FIGURE 56

Thin section of an infected cell showing viral particles stained by the indirect immunoferritin technique using anti-complex E antiserum.

Mature and immature viral particles showing ferritin grains over their outer layers. The cells were fixed in 2.5% paraformaldehyde for 30 min. x 120,000.

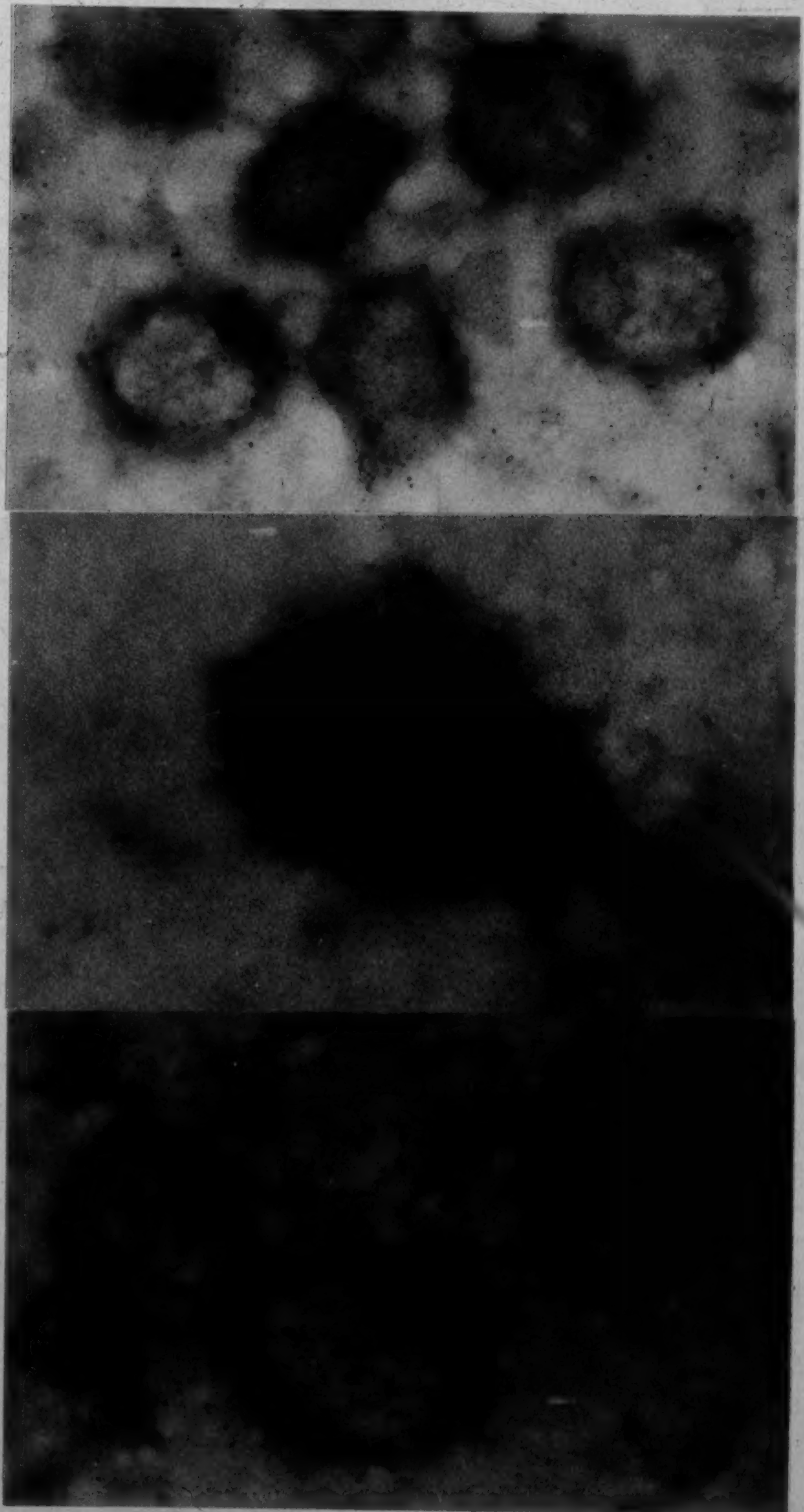


FIGURE 57

Thin section of an infected cell stained by the indirect immunoferritin technique using anti-complex E antiserum.

Low magnification view of an infected cell fixed in 2.5% paraformaldehyde for 30 min. x 25,000.

FIGURE 58

Thin section of an infected cell stained by the indirect immunoferritin technique using anti-complex E antiserum.

High magnification view of the inset of Fig 57 showing a typical distribution of ferritin grains in the cytoplasm and over the viral particles. x 52,000.

FIGURE 59

Thin section of an infected cell stained by the indirect immunoferritin technique using anti-complex E antiserum.

High magnification view of the inset in Fig 57 showing a typical distribution of ferritin grains in the cytoplasm and over the viral particles. x 52,000.

TABLE 5

Ferritin grain counts for control and sections stained with specific antiserum

Sample	Surface examined	Average number of ferritin grains
Formvar	8 μ^2 (1)	0.15 grain/ 0.09 μ^2 (2)
GMA	15.5 μ^2 (1)	5.3 grains/ 0.09 μ^2 (2)
Virus particles (control)	5.5 μ^2 (3)	3.8 grains/ virus particles (4)
Virus particles (stained with specific antiserum)	6 μ^2 (3)	31.1 grains/ virus particles (4)

(1) Determined directly of the electron microscopy negatives.

(2) 0.09 μ^2 = maximum surface of a section of a viral particle in a thin section

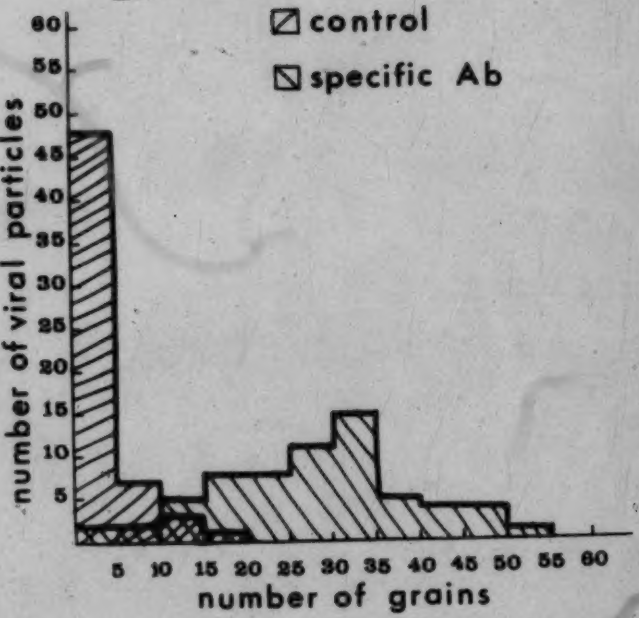
(3) Surface = number of virus particles examined x 0.09 μ^2.

(4) Total number of grains counted over the viral particles/ total number of viral particles examined.

FIGURE 60

Histogram of the ferritin grain distribution for control, and sections stained with specific antiserum.

Electron microscopic negatives of thin sections stained with specific antiserum were examined and the number of ferritin grains over the viral particles was counted using a binocular microscope. A total of 60-65 virus particles was used in each case. The control sections were stained with ferritin conjugate only.


▨ control
▧ specific Ab
number of viral particles
60
55
50
45
40
35
30
25
20
15
10
5
number of grains
5
10
15
20
25
30
35
40
45
50
55
60

DISCUSSION

GROWTH CHARACTERISTICS OF VACCINIA VIRUS STRAIN "WYETH CALF ADAPTED"

The growth characteristics of this strain of vaccinia virus are typical of the vaccinia virus group as indicated by the appearance of the one-step growth curve (Fig 3) and by the total number of PFU's produced by an infected cell (approximatively 50 PFU's) (J.E. Darnell, 1965). The small difference between the total number of intracellular and extracellular PFU's indicates that the extrusion of the virus proceeds rapidly once the immature particles are transformed into virions. As indicated in Figure 3, about 25% of the total PFU's produced are extracellular. Normally, the amount of extracellular PFU's accounts for less than 10% of the total number of PFU's produced by an infected cell (Salzman, 1960). The rapid extrusion is substantiated by personal observations of infected cells examined by electron microscopy. Indeed, the cytoplasm contains few mature particles. Figure 47 shows the disproportion between the two types of viral particles.

As indicated in Table 1, the plaquing efficiency of the viral particles increased rapidly during the passage of vaccinia virus in cell culture. When the ratio of VP/PFU had reached about 10, the virus was then cultured for future work. Furthermore, the number of infectious units produced by an infected cell was about 50; this was found sufficient to produce large quantities of virus in roller bottles.

Table 2 indicates that purification of vaccinia virus by a single cycle of sedimentation velocity in a sucrose gradient gave a yield of 26% (the number of viral particles in the crude extract being 100%). The viral suspension obtained after a single centrifugation in a sucrose gradient was shown to be pure by all three criteria described in Materials and Methods (electron microscopy, absorbance and immunodiffusion). The virus had however suffered some kind of damage during the manipulations. Table 2 indicates that the ratio of VP/PFU increased during the purification step. This increase of the ratio would mean that the plaquing efficiency of the virus suffered a decrease i.e. the virus had been damaged.

While the plaquing efficiency of the virus had been lessened with regards to infectivity, it was necessary to accept this loss to ensure that non-viral contaminants would not intervene with subsequent investigations. On the other hand, the loss of infectivity per se would not pose an obstacle to the analytical studies which will be described.

POLYPEPTIDE COMPOSITION OF VACCINIA VIRUS

Purified vaccinia virus was found to be composed of 33 polypeptides* of different molecular weights. The molecular weight of the polypeptides ranged from 11,000 to 150,000 daltons (Table 3) as determined by SDS gel electrophoresis. The densitometer tracings of the gels have indicated that the VP-2, VP-4's, VP-6's, VP-10 and VP-17 polypeptides were the most abundant in the viral particles. In addition the results showed that the VP-17 polypeptide might be composed of 2 polypeptides. Only gels containing higher concentration of polyacrylamide (15-20%) could tell if indeed VP-17 is actually made of more than one polypeptide.

So far the number of polypeptides composing the virus is the highest reported for vaccinia and for that matter, the highest reported for any animal virus. This number is well within the reach of the nucleic acid of the virus with a molecular weight of 160-180 x 10^6 daltons. A nucleic acid of such a size can code for 200-400 average polypeptides. Furthermore, the structural complexity and the number of enzymes (7) associated with this virus justifies such a high number of polypeptide components.

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* For the present study, it is assumed that each stained band will represent a unique polypeptide i.e. each stained band has a unique composition and sequence.

Our results are in agreement with those obtained by Sarov and Joklik (1972a) and Obijeski et al. (1973). Sarov and Joklik (1972a), utilizing 10% and 15% acrylamide gels and dissociating conditions similar to ours except for the use of 8.2M urea, found a total of 30 polypeptides composing vaccinia virus. The molecular weights of their polypeptides ranged between 8,000 and 250,000 daltons (both limits were estimated). The main difference between the gel pattern of Sarov and Joklik (1972a) and our was that they found that the fast moving polypeptide which corresponds to VP-17, was composed of three polypeptides of molecular weight varying between 8,000 and 12,000 daltons. The difference between their number of polypeptides and ours was caused by the presence of minor polypeptide components of the virus. The amount of each polypeptide present, calculated from gel autoradiograms containing dissociated viral particles, fully labelled with ^{14}C amino-acids, also showed a similarity: VP-2, VP-6's, VP-10 and VP-17 were the most abundant polypeptides.

Our results are also similar to those obtained by Obikeski et al. (1973). Using discontinuous SDS gel electrophoresis and 8% and 10% gel, these authors found 31 distinct polypeptides composing vaccinia virus. The molecular weights of these polypeptides varied between 10,000 and 130,000 daltons. Again, the difference between their gel pattern and ours was found among minor constituants of the virus. The polypeptides corresponding to VP-2, VP-4's, VP-6's, VP-10 and VP-17 were the most abundant.

Even if the results found by the above authors and ours are similar, there are nevertheless some differences. These differences can be explained by the fact that three slightly different techniques of SDS gel electrophoresis were used. Possibly there might be a difference between the various strains of vaccinia virus. These authors have used a mouse neurotropic strain (WR) while our strain was the "Wyeth calf adapted" strain of vaccinia virus. A third explanation for the differences found is that the virus was grown in different cell cultures and if vaccinia virus incorporates host cell material in its structure, it is likely that these components will be different from one cellular system to another. So far, there has not been any definite proof that the virion contains host cell material.

Even if our results and that of other authors indicate a similar but complex composition of polypeptides for vaccinia virus, they were all obtained using SDS gel electrophoresis, as a mean of separating the dissociated polypeptides. This technique has certain limitations:
1) two polypeptides of the same molecular weight, but with different amino acid composition may not be separated,
2) some glycopeptides have been known to give rise to more than one stained band in SDS gel electrophoresis

(Fairbanks et al., 1971), 3) some polypeptides may not bind the dye strongly enough to be detected, 4) some polypeptides may not bind SDS and thus may not penetrate the gel.

Certainly, it would be advantagous to try to separate the dissociated polypeptides by other means. Moss and Rosenblum (1972), have demonstrated the use of hydroxylapatite chromatography for separating polypeptide-SDS complexes.

Regardless of the limitations of ~~the~~ SDS gel electrophoresis technique, the fact that our results obtained with a staining technique agree with those found by Sarov and Joklik (1972a), who had used autoradiography to study the polypeptide composition of the virus, indicates the usefulness of staining polypeptides with Coomassie Brilliant Blue. This dye has been used extensively in this type of work, but it cannot be used for accurate quantitation of polypeptides in SDS gels. At best, it is a very sensitive dye (0.25-0.5 μg of protein can be detected) that can be used only semi-quantitatively. For this reason, quantitation of the viral polypeptides will not be discussed in this work.

Specific staining of the polypeptides of vaccinia virus showed the presence of 8 glycopeptides. Some of these glycopeptides migrated in the VP-4 region, in the VP-6 region and between VP-4 and VP-6. This is in agreement with the results obtained by Holowczak (1970), who found ^{14}C-glucosamine associated mainly with the VP-6 group of polypeptides, and also associated with polypeptides migrating in the VP-4 area and in between the VP-4 and VP-6 group of polypeptides. Another group of polypeptides that gave a positive staining reaction for glycopeptide, migrated on each side of VP-10 and as VP-17. These glycopeptides are reported for the first time.

Other workers have found only 1 or 2 glycopeptides associated with vaccinia virus, when using labelled glucosamine and autoradiographic methods of detection. Sarov and Joklik (1972a) found that both VP-6a and VP-6b contained labelled glucosamine but Garon and Moss (1971) using almost identical experimental conditions found that only VP-6a could be labelled with radioactive glucosamine. Both Sarov and Joklik (1972a) and Garon and Moss (1971), used the mouse neurotropic strain of vaccinia virus (WR strain) but grown in different cellular systems.

The polypeptides containing the most carbohydrates migrated as VP-6a and b and VP-17. As indicated in table 3, their molecular weights are 36,000, 32,000 and 11,000 daltons, respectively. These molecular weights are so far the lowest ones reported for glycopeptides of enveloped viruses. Other glycopeptides associated with enveloped viruses such as the herpesviruses and the myxoviruses have molecular weights in excess of 50,000 daltons (Mountcastle et al., 1971; Eckert, 1973; Spear and Roizman, 1972).

Here again, the use of SDS gel electrophoresis limits the exactitude of the results since some glycopeptides have unusual behavior (aggregation) in dissociative conditions and have irregular mobility in SDS gel electrophoresis (Fairbanks et al., 1971; Segrest et al., 1971; Katzman, 1972). Furthermore, since the intensity of staining depends on the amount of carbohydrate present in the peptide, it is possible that certain glycopeptides were undetected in the gel. Because of the above considerations, it is difficult to ascertain if there are 2 or 8 glycopeptides in vaccinia virus until these molecules are extracted, purified and characterized.

As mentioned in Results, the staining reaction took 5-7 days to fully develop. The reason for the slow color development is not known, but one possible explanation is that the carbohydrate moiety was not readily available to the reagent (fuchsin) and 5 days of incubation was necessary to give rise to the C=0 groups essential for the color development.

Nevertheless, we confirm the presence of glycopeptides in vaccinia virus. It now raises the question whether the carbohydrate content is cell or viral coded. Also, what is the role of these glycopeptides in the virus? Another point to consider is the possibility of non-viral contaminant in the virus. Even if our preparations were pure, as judged by the criteria mentioned in Materials and Methods, there are two possibility of non-viral components associated with the virus. First, non-viral material could be bound so strongly to the viral surface, that the conditions required to dissociate them, might affect the viral structural integrity of the infectivity. Second, because of the procedures of the maturation process, it is possible for non-viral material to be trapped inside the viral envelope. Because viral maturation occurs in "cytoplasmic factories" which contain large amounts of non-structural viral components, it is also possible that some of this material be non-specifically integrated in the virus during the process of maturation.

EXTRACTION OF VACCINIA VIRUS PROTEINS

After having ascertained the polypeptide composition of vaccinia virus by SDS gel electrophoresis, a study of the solubility and of the location of some of these polypeptides was undertaken.

The structural stability of the vaccinia virus is reflected by the fact that extreme mechanical treatment (sonication, French press) failed to disrupt the viral particles. Little protein material was released and electron microscopy revealed structural alterations, only after subjecting the viral suspension to the French press. It was then found that the virus had an increased permeability to sodium phosphotungstate when negatively stained for electron microscopy examination. This resistance of vaccinia virus to mechanical treatment was in contrast to Yaba tumor monkey poxvirus which was partially disrupted by sonication and by French press treatment (Yohn and Olsen 1970).

Treatment of vaccinia virus with guanidine hydrochloride, urea and high pH (10.5) indicates that vaccinia virus is chemically quite stable. Only 2 polypeptides are released in any significant amount: the VP-17 and another one migrating between VP-6 and VP-4. The results also indicate that most of the surface polypeptides of the virus are strongly bound to the virus. If none of the polypeptides extracted by the various treatments came from inside the virus, then it can be assumed that most surface polypeptides (8-14) have a molecular weight between 11,000 and 70,000 daltons, since most of the polypeptides migrated between VP-4 and VP-17. Only treatment of the virus at pH 10.5 released some VP-2. In conclusion, it can be said that there exists a great diversity of polypeptides on the surface of vaccinia virus. The chemical stability of the virus is reflected by the fact that very little of the surface polypeptides were released by 8M urea, 6N guanidine hydrochloride and high pH (10.5): this would also indicate that most of these polypeptides are held onto the viral surface by bonds stronger than hydrogen bonds.

Our results generally agree with that of Sarov and Joklik (1972a), who found a total of 14 polypeptides associated with the outer layer of the virus. Most of them were of low molecular weights: only 4 of 14 were in excess of 40,000 daltons. Our results are also in agreement with those of Katz and Margalith (1973), who found surface polypeptides migrating with VP-2, VP-4's, VP-6's, VP-10 and VP-17.

Because these treatments either denatured the extracted proteins, or too little protein material was extracted, it was decided to use detergents. Most of the non-ionic detergents released a small amount of protein or none at all. Such was the case with saponin, Brij 35 and 58, Tween 60 and 80.

As shown in Table 4, the ionic detergents were more satisfactory in solubilizing viral protein, because upon reacting with a protein, the latter bears a strong ionic charge, thus facilitating solubilization. The relationship between the amount of protein released and the type of detergent was an inverse relationship between the quantity of protein extracted and the molecular weight of the detergents. Sodium dodecyl sulfate has the lowest molecular weight (288) and is the best solubilizing agent while NP-40 has the highest molecular weight (602) and extract only 26% of the total protein content of vaccinia virus. The fact that the detergents were not equimolar with respect to each other during the extraction, cannot explain the inverse relationship since higher concentrations of detergents or longer incubation periods did not increase significantly the amount of protein solubilized. One possible explanation is that structural hindrance by the viral envelope or the surface protein coat might prevent further extraction by the higher molecular weight detergents. Indeed, when a disulfide bond reducing agent is used, there is an increase in the amount of protein extracted by SDC and NP-40. If the reducing agent can loosen up the outer layer of the virus, more protein could be extracted by the detergents. This explanation is not unlikely, since there is no significant increase in the amount of protein released when CTAB is used with or without a reducing agent. Electron microscopy examination of the viral particles after treatment with CTAB alone, shows that the surface protein coat and the viral envelope are extracted by the detergent. Thus there would be no effect on the two structural components of the virus, by the addition of a disulfide bond reducing agent.

None of the detergents was completely selective in its action as indicated in Figures 9, 10 and 11. The most "selective" of all was NP-40 which solubilized 11 polypeptides from the virus. Among them were 6 glycopeptides, including VP-6a and VP-6b. This detergent (NP-40) has already been shown to preferentially solubilize glycoproteins from herpes simplex virus (Spear and Roizman, 1972). As reported before (Joklik and Holowczak, 1967a), the most abundant polypeptides released are the VP-6 group. Furthermore, material migrating as VP-2, the VP-4 group, VP-10 and VP-17 are also released in smaller amounts. Similar results were also found by Sarov and Joklik (1972a). Using fully labelled virus, these authors found that NP-40 extracted a total of 8 polypeptides and all of them were found to be located in the outer layer of the virus. Our SDS gel electrophoresis pattern of the extracted polypeptides was quite similar to theirs, except for three high molecular weight polypeptides migrating as VP-2 and VP-4.

Not only did the ionic detergents release large quantities of proteins, they also solubilized most of the viral polypeptides (Fig. 10 and 11). SDC extracted less protein than CTAB but only a few minor polypeptides present in the CTAB extract, were not detected in the SDC extract. The VP-6 group of polypeptides is a major component released in all cases, except for SDC and CTAB treatments where a large amount of the VP-4 group was also extracted. From Figure 11, it seems that VP-2, the VP-4 group and VP-17 should be the only major polypeptide component left after CTAB treatment.

ANALYSIS OF VACCINIA VIRUS AFTER EXTRACTION BY DETERGENTS

The SDS gel electrophoresis analysis of vaccinia virus after NP-40 extraction shows that all the polypeptides composing the virus were present. Only a reduction in the amount of the VP-6a and b could be noticed. This reduction in the VP-6 group could not be estimated because of the use of Coomassie Brillant Blue to stain the polypeptides. Earlier workers had suggested that NP-40 could remove all of the VP-6 group (Holowczak and Joklik, 1967a). The fact that none of the kind of polypeptides extracted by NP-40 was totally released from the virus could be due to a strain difference as suggested earlier. Recently, Spear and Roizman (1972) found that NP-40 would extract mainly glycopeptides from herpes simplex virus, but the extraction was never complete in that of all the different polypeptides extracted, none were completely released from the virus.

The SDS gel electrophoresis pattern of the solubilized virus after SDC extraction is strikingly similar to that of Figure 6 which shows the gel pattern of virus completely solubilized in SDS. Since most of the polypeptides composing the virus are released by SDC and most types of polypeptides are found associated with the extracted viral particles, the indications are that SDC can partially solubilize polypeptides from anywhere in the virus or that all types of polypeptides are accessible on the viral surface or the purified viral preparations are heterogeneous.

The SDS gel electrophoresis pattern obtained from solubilized viral particles after CTAB extraction shows that the extracted particles were composed mainly of the VP-4 group of polypeptides plus most of the higher molecular weights polypeptides. Little material of molecular weights between 11,000 and 70,000 daltons was not solubilized by CTAB, except for some VP-17. Since the viral particles extraction by CTAB are devoid of their viral surface coat and viral envelope (Figs 35 and 36) these results confirm the fact that the surface of the virus is composed mainly of polypeptides with molecular weights ranging between 11,000 and 70,000 daltons. The VP-17 polypeptide is extracted by all three detergents, but not completely since it is found associated with the extracted viral particles. Possibly this could suggest that actually VP-17 is composed of 2 polypeptides as it was seen on a few gels (Figs 6, 10 and 11); one would be extracted by all the detergents while the other would not.

From the gel electrophoresis analysis of the chemical and detergents extracts combined with the gel electrophoresis analysis of the extracted viral particles, the following points can be concluded: a) there is a large number (8 to 14) of different size polypeptides on the surface of vaccinia virus; b) most of the surface polypeptides have a molecular weight varying between 11,000 to 70,000 daltons; c) the polypeptides composing the inner region of the virus have a molecular weight varying between 70,000 and 150,000 daltons d) none of the three detergents used in this study was selective i.e. all kinds of polypeptides were extracted, but none were completely extracted e) NP-40 extracted most types of glycopeptides from the virus, but none were completely extracted from the virus; f) dithiothreitol increased the amount of protein extracted by SDC and NP-40, but the same type of polypeptides as were extracted by the detergents alone were solubilized in the presence of the disulfide bond reducing agent.

The analysis of the extracted viral particles by electron microscopy substantiated the results obtained by SDS gel electrophoresis. The detergent NP-40 did release material from the surface of the virus. Both negative contrast staining and ultramicrotomy failed to indicate morphological alterations to the inside of the virus. In contrast the appearance of the surface and the viral envelope was markedly altered. The envelope became more permeable to the negative stain, while the surface had lost parts of its surface coat. This would indicate that the material extracted by NP-40 may be localized or concentrated in packets on the viral surface.

The increase in permeability to the negative stain has permitted a better visualization of the core content. In the occasional particles, one could see the tubular "S" shaped organisation of the nucleoprotein complex of the virus. This "S" shaped organisation of the nucleoprotein complex was also seen in viral particles after CTAB treatment. These findings substantiate the early work of Peters and Müeller (1963) and Peters and Büttner (1965), who had also found a tubular organisation inside the viral core of vaccinia virus. These results would indicate that the nucleic acid of the virus is not in a free state in the core, but is combined most probably to protein material acquiring a distinctive tubular appearance and packaged in an orderly fashion inside the viral core.

Examination of viral particles by negative contrast staining indicates that two distinctive types of alterations were caused by SDC. Each of these types of alterations can be related to the changes seen by ultramicrotomy. Surface alterations are present and core alterations are seen only if the viral envelope is loose enough to let the negative stain penetrate the viral particles. The surface protein coat of the virus has been removed so that one looks directly at the viral envelope (Fig. 25). The other kind of change indicates that the surface of the virus has been altered enough to let a portion of the viral core be extracted by the detergent. Both negative contrast staining and ultramicrotomy show this kind of alteration: the core content has taken a shrunken or coagulated appearance (Figs 23 and 24).

In conclusion, it can be said that SDC can cause at least two different types of alteration of the ultrastructure of vaccinia virus: one affects the viral surface and the other affects the viral surface and the core content.

The results obtained after extraction by CTAB supports the findings of SDS gel electrophoresis analysis: core material is not well solubilized by the detergent. Also, Figures 35 and 36 show the "S" shape organization of the nucleoprotein complex of the virus. The residual material after extraction with CTAB had the appearance of membranes. Figures 35, 36 and 37 show these structures and the absence of staining material left inside the core of the virus. Not only the surface coat and the viral envelope were solubilized but also material from the core, the core envelope and the lateral bodies. Not all the lateral bodies were extracted as indicated in Figure 38; about 10% of them were still attached to the viral core. What seems to be left after extraction by CTAB, is the backbone structure of the core of the virus. Most probably, it represent the structural organization of the high molecular weight polypeptides composing the viral core (core envelope and the nucleoprotein matrix).

From the combined results of SDS gel electrophoresis of detergents extracts and of the extracted viral particles, plus the examination of these particles by negative contrast staining and ultramicrotomy, two hypothesis can be submitted: a) when vaccinia virus is purified by sedimentation velocity in a sucrose gradient, the resulting population of virions is heterogenous in such a way that the viral particles will be affected to a different degree by some detergents; b) there are evidences that suggest that certain polypeptides may be located in the outer and in the inner layer of the viral particles i.e. certain polypeptides are not restricted to a specific structural component in vaccinia virus.

Electron microscopic examination of the viral particles after extraction by the detergent SDC shows an example of this heterogeneity. Only a difference in the viral particles can account for the alterations seen by negative contrast staining and ultramicrotomy. In the case of SDC, all the particles had lost their surface protein coat and 60% had an altered core. This would indicate that some particles after the loss of their surface coat became more permeable, so that core material could be extracted or at least affected by the detergent. As mentioned earlier, longer incubation periods or higher concentrations of SDC did not solubilize any more material: this would suggest that SDC altered preferentially a part of the purified viral population. Possibly this population indistinguishable from the more resistant one by electron microscopy, would posses a viral envelope somewhat more permeable so that its core content could be affected by SDC.

This heterogeneity of purified vaccinia virus has already been reported by Sharp and McGuire (1970) and McGuire and Sharp (1972). These authors have shown that when vaccinia virus was sedimented in a sucrose gradient, the fast sedimenting particles had a lower plaquing efficiency than the slow sedimenting particles. Furthermore, these authors found that vaccinia virus where sedimented in a shallow gradient of potassium tartrate showed the presence of at least two populations having different densities. Heterogeneity has also been found among other viral population. Nonoyama et al. (1972) have found that defective virus were present among a population of reovirus purified by isopycnic centrifugation in cesium chloride. These defective virus particles could be detected only after treating the purified suspension with chymotrypsin. E. Norrby (personal communication) has recently found that a purified suspension of adenovirus contained more than one kind of virions. These virions having a very small density difference in the presence of heavy salts were found to be different in their susceptibility to tryptic digestion.

The second hypothesis concerning the location of some of the structural polypeptides in vaccinia virus is in disagreement with the idea of Sarov and Joklik (1972a), in which each structural polypeptide has a unique and distinct location within the viral structure. Among the evidence suggesting such a hypothesis is the fact that the use of DTT increases the amount of protein extracted by SDC and NP-40 but not the number of polypeptides extracted by detergent alone. Since in the case of SDC, electron microscopy has shown that most of the surface material is gone, then the extra amount of polypeptide must have come from the inside of the virus. If some polypeptides can be extracted from the surface of the virus and from other parts of the virus, then one must assume that they were located in different structural components of the viral particles.

One alternative to this hypothesis is that detergents can remove polypeptides from anywhere in the virus without causing alterations detectable by electron microscopy.

Because of the limitations of the techniques utilized in this work and of the large number of polypeptides composing the virus, it is very difficult to say which of the polypeptides are associated with the inner and the outer regions of the virus. From the SDS gel electrophoresis results, it would seem that the most abundant polypeptides are located in different regions of the virus. The polypeptides responsible for the structural organization of the nucleoprotein complex and responsible from the various enzyme activities associated with the virus, are most likely located in a specific region of the virus. There are no apparent reasons why the other structural polypeptides should have a unique location in the viral particles, as stated by Sarov and Joklik (1972a).

This hypothesis is supported by the work of Downer et al. (1973) and Kleiman and Moss (1973), who have found viral phosphoproteins of molecular weight around 11,000 daltons associated with the surface and with the core of vaccinia virus. Furthermore, Garon and Moss (1971) and Sarov and Joklik (1972a and 1972b) found surface polypeptides associated with viral cores. They considered that this association was due to incomplete removal of the outer layer of the virus or to readsorption of surface polypeptides after solubilization by detergents. Recently, Poliski and Kates (1972) found that proteins were firmly associated with replicating viral DNA during infection. Some of these proteins migrated in SDS gels as structural polypeptides of vaccinia virus. Among those polypeptides were the VP-6's and other small molecular weights polypeptides normally associated with the outer layer of the virus. This finding supports the idea that some structural polypeptides are located in the inner and in the outer layers of vaccinia virus.

PURIFICATION AND LOCALIZATION OF COMPLEX E IN VACCINIA VIRUS

Previous analysis of the NP-40 extract of vaccinia virus had shown its composition to be simple. One major antigen was present in the extract, as shown in Figure 16. After attempted fractionation by column chromatography, tests were conducted to determine the purity of the complex E peak. Microimmunodiffusion and immunoelectrophoresis showed the preparation to be devoided of major antigenic contaminants. Since in both cases, the amount of complex E analysed contained about 25 μg of protein and these techniques can detect less than 0.5 μg of antigen (Kwapinski, 1965), this would indicate that the preparation was quite pure with respect to antigens precipitable by antisera against whole vaccinia virus and against host cell antigens. Analytical ultracentrifugation also showed the presence of a single component in the complex E peak. Although this technique is not sensitive to detect minor contaminants, (0.5 mg/ml of protein are needed to produce an adequate peak) it could not detect any contaminant having sedimentation properties different to that of complex E. Since up to 2 mg/ml of complex E were analysed by ultracentrifugation and that no other peak were detected, it would indicate that the complex E peak was fairly pure.

Both discontinuous gel electrophoresis and SDS gel electrophoresis, combined with staining of polypeptides with Coomassie Brilliant Blue, are techniques that can detect up to 0.25-0.5 μg of protein. Unlike the immunodiffusion and immunoelectrophoresis techniques, they do not depend on the formation of a precipitin band, but solely on the presence of protein material. Thus these techniques can be regarded as very sensitive to detect minor contaminants. The results obtained with the SDS gel electrophoresis technique must be interpreted with caution because SDS dissociates proteins into sub-unit constituents. Furthermore, the material extracted by NP-40 was mainly composed of glycoprotein material and as mentioned earlier, they can exibit atypical behavior such as variable mobility in SDS gel and random aggregation in presence of SDS (Bretscher, 1971; Fairbanks et al., 1971; Segrest et al., 1971; Katzman, 1972). Repeated electrophoresis of an NP-40 extract in SDS gave about 10-11 polypeptides bands. In many cases, the VP-6b band was not seen and instead, a stronger VP-6a would be present. This was probably caused by the improper separation of the VP-6 polypeptides. Nevertheless, the results indicate that the material purified by chromatography contained three major polypeptides of vaccinia virus; the VP-6's and VP-10 of molecular weight of 36,000, 32,000 and 22,000 daltons respectively. The same caution must applied to the results of discontinuous gel electrophoresis. As indicated in Figure 43, the complex E peak contained one major stained band. The small band migrating ahead represent either a true contaminant, or an artifact caused by the nature of the extracted material (glycoprotein).

Since 200 µg of protein were applied onto the gel, this small band, if a true contaminant, must represent a small percentage of the total amount of protein present in the complex E peak.

Because of the above considerations the preparation was judged pure enough for further work, and antibodies against complex E were produced in rabbits as described in Material and Methods. The antisera obtained gave a single precipitin band when tested against a fresh NP-40 extract and against a viral soluble fraction which contained complex E, free of detergent. Since up to 200 µg of antigen were tested against the antisera, this would indicate that the antisera were monospecific to the limit of the test.

Evidence from gel electrophoresis and electron microscopy indicated that complex E was located in the outer layer of the virus. When antisera were tested for their ability to neutralize the virus, it was found that indeed they could prevent the infection of susceptible cells by vaccinia virus. If antibodies were directed against material situated anywhere else than the outer layer of the virus, neutralization would be hard to imagine. If the neutralization of the virus can be caused by antibodies against complex E, this would strongly suggest that this glycoprotein is situated on the surface of the virus, or at least situated at a point where antibodies can attach. Since neutralizing antibodies normally prevent attachment of the viral particles to the host cell membrane or prevent the uncoating of the virus inside the cell, these results would suggest that: a) complex E is situated close to the attachment site on the virus, so that antobidies bound to complex E would prevent attachment to the host cell by steric hindrance; b) complex E upon reacting with its antibodies would cause a conformational change of the viral surface, so as to prevent attachment of the virus to the host cell or prevent uncoating of the virus; c) complex E is the structure responsible for the attachment of the virus to the host cell and infection is prevented when antibodies attach to this protein. The neutralization of vaccinia virus by anti-complex E serum indicates that most likely this protein is located in the outer layer of the virus. Because electron microscopy examination of viral particles after NP-40 treatment has indicated that both the surface protein coat and the viral envelope had been altered by the detergent, it cannot be said whether complex E is located in the viral envelope or in the surface protein coat. The most likely location for complex E would be in the surface coat, since it is the most altered structure after NP-40 treatment while the viral envelope appears intact when examined by ultramicrotomy. Furthermore, it would appear much easier for antibodies to react with an antigen situated in the surface coat than with the viral envelope, which is located underneath the surface protein coat.

The results obtained agree with the earlier work by Cohen and Wilcox (1966), who had found that the antigen(s) responsible for giving rise to neutralizing antibodies against vaccinia virus had a molecular weight of 100,000 to 200,000 daltons. Gel chromatography and gel diffusion results suggest such a size for complex E. It is excluded by Sephadex G-100, but not by Sephadex G-200 and furthermore the straight precipitin line obtain by immunodiffusion suggest a molecule with similar diffusing properties as gamma-globulins (M.W. of 150,000 daltons).

Many viral glycoproteins have been implicated in the induction of neutralizing antibodies. Ponce de Leon et al. (1973) found that a herpes simplex virus glycoprotein could induce the formation of neutralizing antibodies. This glycoprotein is in the process of being purified and characterized. The glycoproteins of the myxovirus family have been studied extensively. The glycoprotein of influenza virus(M.W. of 48,000 daltons) and a vesicular stomatitis virus were purified and both of them could give rise to neutralizing antibodies (Kelly et al., 1972; Eckert, 1973). In the case of the glycoprotein of vesicular stomatitis virus, only this antigen could give rise to neutralizing antibodies; the other antigens composing the virus could not.

So far most enveloped viruses have been shown to posses a surface glycoprotein and for a few of them it has been shown that this glycoprotein was responsible for giving rise to neutralizing antibodies. For the influenza viruses, it has been shown that removal of the carbohydrate portion of their glycoproteins would prevent their attachment to the cell surface. It seems then, that glycoproteins of enveloped viruses are indeed responsible for their attachment onto the host cell membrane. As far as the glycoprotein of vaccinia virus is concerned, it is not known whether or not only antibodies against complex E can neutralize the virus. It would be interesting to find out if antiserum produced against whole vaccinia virus could still neutralize the virus after exaustive absorption with purified complex E.

The immunoferritin results confirm that complex E was located in the outer layer of the virus. The number of grains and the distribution over the viral particles as indicated in Table 5 and Figure 60 is 8 times higher than over the controls. The fact that 31 grains as an average are located over the viral particles and the appearance of the distribution curve in Figure 60 are indications that some of the complex E molecules were denatured during the fixation and the embedding procedures. Since the amount of denaturation is not known, it cannot be concluded that complex E is present only in the outer layer of the virus, even if no concentration of ferritin grains was ever seen located over the inner layer (core, core envelope and lateral bodies) of the virus.

Because of the size of the conjugate and the use of the indirect staining technique, the resolution is approximately 40 nm. Thus, it may be possible to say that complex E is situated in the outer layer of the virus, but one cannot determine if the glycoprotein is located in the surface coat or in the viral envelope.

Because of the large difference in the morphology and in the composition of the immature forms of the virus, ferritin grains were not counted over these immature particles. Nevertheless, observations by electron microscopy has indicated that the immature forms had ferritin grains located over their outer layer (see Figs 57, 58 and 59). Because of the extraction effect discussed before, and also because of the poor preservation of the ground cytoplasm, no ferritin grain counts were made in the cytoplasm. Nevertheless, very few grains were found associated with the host cell membrane suggesting that complex E is not a viral antigen appearing on the host cell membrane during infection (Ueda et al., 1969).

In conclusion, the combined results of detergent extraction by NP-40, electron microscopic observations, neutralization experiment and immunoelectron microscopy, indicate that complex E a major glycoprotein antigen of molecular weight between 100,000 to 200,000 daltons, is located in the outer layer of vaccinia virus. This antigen may play a major role in the attachment of the virus to the host cell membrane since it can give rise to neutralizing antibodies.

Our results are in agreement with the recent articles concerning the number of polypeptides composing the virus and also the localization of the low molecular weights polypeptides.

Our SDS gel pattern (Fig. 6) resembles the one presented by McCrae and Szilagyi (1975), except that these authors had an increased resolution due to the use of an SDS discontinuous gradient gel electrophoresis system. Our results also agree with the work of Pogo et al. (1975) and Kleiman and Moss (1975) which suggested the presence of the low molecular weight polypeptides (11,000 - 12,000 daltons) in the core and in the outer layer of the virus.

To make it easier to the reader, a short summary of the correspondence in the numbering of the polypeptides between our work and that of Sarov and Joklik (1972a) and of McCrae and Szilagyi (1975) is presented:

1) the groups of VP-2, VP-4 and VP-6 are the same in all cases.
2) our VP-10 corresponds to the VP-8 of Sarov and Joklik (1972a) and to the VP-8 of McCrae and Szilagyi (1975).
3) our VP-17 corresponds to VP-11 and VP-12 of Sarov and Joklik (1972a) and to VP-11 of McCrae and Szilagyi (1975).

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